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Vol. IV. No. 5] [October, 1923.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, January to December, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

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THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

VARIATION PHENOMENA IN STREPTOCOCCI: FURTHER STUDIES ON VIRULENCE AND IMMUNITY.

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Received for publication June 14th, 1923.

In a previous communication (Cowan, 1922) it has been stated that from individual strains of streptococci two types of organisms can be "dissociated"; these have been called "rough" (R) and "smooth" (S) types. The former grows as a sediment in broth and shows dense colonies with irregular outline on agar, while the organisms are large and appear in bunches or chains; the latter render broth turbid and form more translucent colonies with regular outline on agar, while the individual cocci are smaller and show fewer bunches and chains. The most important difference found was, however, that of virulence, the "S" type being markedly more virulent than the "R" type.

In the following communication are recorded further experiments designed to elucidate more precisely the characteristics and relationships of these two types of organisms.

VIRULENCE.

It had been found that the "S" type of streptococcus was much more virulent than the "R" type when the organisms were injected intraperitoneally and subcutaneously into mice and when given intravenously to rabbits. The following experiments were performed in order to determine whether the same difference could be elicited when they were given to mice intravenously.

Exp. 1.—$\frac{1}{4}$ c.c. of a 24-hour serum broth culture of the "R" and "S" types of each of 7 strains of hæmolytic streptococci was given intravenously to mice—the tail vein being used. In each case 2 mice were used, so that 14 mice received the "R" type, and 14 mice received the "S" type of organism.

Result.—At the end of 6 days, 9 mice given "R" type and 12 mice given "S" type were dead.

Exp. 2.—$\frac{1}{20}$ of a 24-hour growth on a serum agar slope of the same "R" and "S" types used in Exp. 1 was injected intravenously into mice, in duplicate.

Result.—At the end of 7 days, 10 of the 14 mice receiving "R" and all (14) of the mice receiving "S" type were dead.

Exp. 3 was a repetition of Exp. 2, except that $\frac{1}{25}$ of the growth from a serum agar slope was used in place of $\frac{1}{20}$.

Result.—At the end of 8 days, 5 of the 14 mice receiving the "R" type and 10 of the 14 mice receiving "S" type were dead.

(Death of a mouse more than 8 days after injection was not considered as due to the injection.)

It would seem from these experiments that if large doses of "R" and "S" types of streptococci are given intravenously to mice, the difference in the mortality, although evident, is not marked. When the dose decreases, however, the difference becomes more prominent.

IMMUNITY TO THE "S" TYPE CONFERRED BY INOCULATION WITH THE "R" TYPE.

A. *Homologous Protection.*

An indication had been obtained (Cowan, 1922) that a dose of the live "R" type would protect a mouse from a lethal dose of the "S" type administered later. The following experiments were performed with a view to verifying and amplifying this finding.

Exp. 1.—"R" type of strain "B.C." was grown on serum agar slopes and an emulsion made in normal saline. Doses of 1, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ of a slope were injected intraperitoneally into mice in duplicate, so that 12 mice received "R" organisms. At the end of 14 days 10 of the 12 survived, and these and one control were each given intraperitoneally 1 m.l.d. of the "S" type of strain "B.C."

Result.—The control and 3 treated mice died overnight while the remaining treated mice were alive and well in 9 days. The treated mice all showed abscesses in the peritoneal cavity.

Exp. 2.—6 mice were each given intraperitoneally $\frac{1}{6}$ of the 24-hour growth from a serum agar slope of the "R" type of strain "B.C." Of these, 4 were alive in 8 days, and each with 1 control was given intraperitoneally 1 m.l.d. of the "S" type of strain "B.C."

Result.—The control mouse died overnight while the treated mice were all alive and well in 9 days.

These experiments were repeated with strains "Ludwig" and "2716." Table I gives the number of mice which received the "R" type, those surviving this, and those which survived a later m.l.d. of the homologous "S" type—that is, those with an immunity produced by the "R" type against the "S" type.

TABLE I.

Strain.	Number of mice given "R" type.	Number of mice surviving and given "S" type.	Number of mice surviving and apparently immune to "S" type.
"B.C."	18	14	11
"2716"	18	11	8
"Ludwig"	23	16	8
	59	41	27

Thus 27 of 41 mice had acquired an immunity against the virulent "S" type by a previous intraperitoneal injection of the homologous "R" type.

B. *Cross Protection.*

The mice which had received the "R" type of streptococci and later survived a m.l.d. of the corresponding "S" type were given a m.l.d. of the "S" type of another strain. A considerable number of these survived, indicating that one strain produced an immunity against another. The following experiments were performed in order to determine whether this immunity was due to the primary dose of "R" organisms.

Exp. 1.—6 mice were each given intraperitoneally $\frac{1}{6}$ of the growth from a serum agar slope of the "R" type of strain "B.C." Eight days later 4 were given intraperitoneally the "S" type of strain "Ludwig" and two were given the "S" type of strain "2716": controls were inoculated with each strain.

Result.—All controls died overnight.

One given strain "2716" died on injection and the other survived. 1 given strain "Ludwig" died in three days and 1 in 4 days, while 2 survived. All the treated mice showed abscesses in the peritoneal cavity.

The experiment was repeated with the "R" type of strains "2716" and "Ludwig." Table II gives the number of mice used in each case and the number surviving.

TABLE II.

Strain.	Number of mice given "R" type.	Number given "S" type of strain "B.C."		Number given "S" type of strain "2716."		Number given "S" type of strain "Ludwig."	
		Died.	Survived.	Died.	Survived.	Died.	Survived.
"B.C."	6	—	—	1	1	2	2
"2716"	4	0	2	0	0	2	0
"Ludwig"	4	0	2	2	0	—	—
	14 : 7 died; 7 survived.						

There is evidently some cross-protection of mice whichever "R" type is given and later the "S" type of another strain; but the number of mice inoculated with each strain and the number of strains used is too small to draw any conclusion as to a relationship of different strains with respect to production of cross-protection.

"LOCAL" OR "GENERAL" CHARACTER OF PROTECTIVE MECHANISM.

It seemed possible that the immunity conferred on mice by the "R" type of streptococcus against the "S" type might be a local one, as, in each case, the two types had been given intra-peritoneally. In order to obtain some evidence for or against this possibility, experiments were carried out in which the "R" type of organisms were given intra-peritoneally and the "S" type intravenously and *vice versâ.*

Exp. 1.—6 mice were each given $\frac{1}{6}$ of the growth from a 27-hour-old serum agar slope of "R" type of strain "B.C.," which was given intra-

peritoneally. In 8 days all were alive, and these and 3 controls were given 1 m.l.d. of "S" type of strain "B.C." intravenously.

Result.—4 treated mice died almost immediately, some with convulsions. The controls and 2 remaining treated mice died within 24 hours. All the treated mice showed abscesses in the peritoneal cavity.

Exp. 2.—Repetition of Exp. 1.

Result.—Again 4 of the 6 treated mice died almost immediately after injection of the "S" type; some had convulsions. One died in 2 days and 1 was alive and well in 10 days.

The almost instantaneous deaths of these treated mice, when given the "S" type of organisms intravenously, pointed to anaphylaxis as a possible cause. To overcome this difficulty the experiment was repeated, but a desensitising dose was given intra-peritoneally before the intravenous dose.

Exp. 3.—6 mice were each given intra-peritoneally $\frac{1}{6}$ of the growth from a serum agar slope of the "R" type of strain "B.C." After 7 days they were each given a very small dose of the same type intra-peritoneally. The next day they were given 1 m.l.d. of the "S" type intravenously.

Result.—The control mouse died in 4 days and all the treated ones survived. 1 of the treated mice when killed after 12 days had pus in the joints of one foot from which a hæmolytic streptococcus was recovered.

Exp. 4.—Repetition of Exp. 3 with the strain "Ludwig."

Result.—All the treated mice and the controls died within 24 hours after receiving the "S" type intravenously. None died immediately after injection and the treated mice did not show abscesses in the peritoneal cavity.

Exp. 5.—Repetition of Exp. 3 with the strain "2716," 4 mice being used.

Result.—1 treated mouse died immediately after injection of the "S" type, but had no convulsions; 1 died in 24 hours; the control died in 3 days; the remaining 2 treated mice survived.

Exp. 6.—6 mice were each given intravenously $\frac{1}{20}$ of the 24-hour growth from a serum agar slope of the "R" type of strain "B.C." After 14 days all were alive, and each and 1 control was given intraperitoneally 1 m.l.d. of the "S" type of strain "B.C."

Result.—4 treated mice and the control died overnight; 1 treated mouse died in 2 days and 1 in 8 days.

Exp. 7.—Repetition of Exp. 6. 4 mice survived the intravenous dose of "R" type, and these and 1 control were given intra-peritoneally 1 m.l.d. of "S" type of strain "B.C."

Result.—All the mice died within 24 hours.

Of the experiments detailed above, No. 3 and No. 5 indicate that the immunity against the "S" type of streptococcus induced by a previous intra-peritoneal injection of the "R" type is not local only. The fact that the mice treated with the "R" type of strain "Ludwig" (Exp. 4) had apparently no immunity may be in some way connected with the fact that no abscesses were found in the peritoneal cavity.

In Experiments 6–7 the dose of the "R" type of organisms was very small, and its failure to give definite immunity against the "S" type was to be expected.

PROTECTION INDUCED IN MICE BY HEAT-KILLED ORGANISMS.

Exp. 1.—6 mice were each given, intra-peritoneally, $\frac{1}{8}$ of the growth from a serum agar slope of the "R" type of strain "B.C.," the suspension having been heated to 56° C. for 1 hour. After 10 days each of these mice and a control was given intra-peritoneally 1 m.l.d. of the "S" type of the same strain.

Result.—The control died within 24 hours. 2 treated mice died in 2 days and 2 in 3 days, while 2 survived.

None of the treated mice which died showed abscesses, but the survivors when killed in 10 days had definite abscesses in the peritoneal cavity.

Exp. 2.—Repetition of Exp. 1 with the preliminary injection of killed "S" type of organisms in place of killed "R" type.

Result.—The control died within 24 hours after the injection of the test m.l.d. of living "S" type; 4 treated mice died in 2 days while 2 survived. The treated mice which died showed no abscesses, but the survivors when killed had abscesses in the peritoneal cavity.

There is evidently some protection acquired by mice against the "S" type of streptococcus, by previous injection of killed organisms, of both the "R" and "S" type. Here, too, there may be some association of protection with abscess formation.

EXAMINATION OF ABSCESSES FOUND IN THE PERITONEAL CAVITY OF MICE AFTER INJECTION OF "R" TYPE OF STREPTOCOCCUS.

A number of mice were each given, intra-peritoneally, 0·5 c.c. of a 24-hour serum broth culture of the "R" type of strain "B.C." These were killed in 3, 5, 7, 10 and 17 days and the abscesses found in the peritoneal cavities were examined histologically.

In all cases there was a definite localisation of the inflammatory process. The 3- and 5-day abscesses were in most part made up of masses of organisms and polymorphonuclear leucocytes. In the older ones the number of small round-cells increased and the number of organisms decreased. In the 10-day-old abscesses fibroblasts could be distinguished at the margin of the abscesses, and in 17-day specimens there was active proliferation of the fibroblasts around and into the abscess. This condition was found in both large and small abscesses although more marked in the smaller ones.

Cultures made from abscesses found in mice from 3 days to 2 months after the intra-peritoneal injection of "R" type of streptococcus have always given a growth of hæmolytic streptococcus of the "R" type.

DISCUSSION.

The results here recorded of the effect of intravenous injection into mice of the "R" and "S" types of streptococcus confirm the findings previously reported, that there is a definite relationship between virulence and the "S" type of organism in a strain of streptococcus.

There is also no doubt that the "R" type when given intra-peritoneally to mice excites the production of immunity against the "S" type. From the fact that mice are protected against the "S" type when given either intravenously or intra-peritoneally, it would appear that the immunity is of a general character, and is not confined to the peritoneal cavity where the "R"

type of organisms was given, and where, in most cases, they persisted for some time in localised abscesses. There would appear to be some association between the occurrence of abscesses and the acquisition of immunity, for mice which proved immune invariably showed abscesses in the peritoneal cavity. A minority only of mice treated and found not to be immune showed these abscesses. It is probable that the abscesses found were due to the initial dose of "R" organisms and not to the test dose of "S" organisms, as they yielded "R" cultures and as the "R" type does form abscesses in the majority of cases; the "R" type of strain "B.C." has never failed to do so if given in such doses as used in these experiments.

Protection of mice against the "S" type of heterologous strains of streptococci is apparently given by injection of the "R" type into the peritoneal cavity. The experiments recorded show some difference with respect to the immunity produced by one "R" type against two other "S" types, but the number of strains used and the number of mice in each series is too small to afford any clue as to whether a grouping of the hæmolytic streptococci may be made on such a basis. They do, however, raise the question of that possibility.

It is proposed to repeat on a larger scale those experiments which have indicated a general immunity as opposed to a local immunity and also those dealing with cross-protection, in order to compare the numbers of mice so protected with the numbers given in Table I of this paper. Also, it will be of interest to determine, over a large series of mice, whether preliminary doses of heat-killed "R" or "S" types are as effective as the living "R" type in producing an immunity against the living virulent "S" type.

SUMMARY.

(1) The "S" type of streptococcus is more virulent for mice than the "R" type when both are given intravenously.

(2) An injection of "R" type organisms gives considerable protection to mice against a subsequent lethal dose of the "S" type even when the test dose is administered by another route. The immunity is, therefore, not purely local, nor is it confined to the "S" type of the homologous strain of the immunising "R" type.

(3) Heat-killed "R" and "S" types in some cases produce immunity in mice against the "S" type.

(4) The "R" type of organisms, when injected intra-peritoneally into mice, localise in abscesses which tend to heal by fibrosis.

(5) There would appear to be an association of immunity with the presence of these localised abscesses.

I wish to thank Prof. Ledingham for much helpful advice and criticism in the course of this work.

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THE SPLEEN AND IMMUNITY REACTIONS TO JENSEN'S RAT SARCOMA.

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Received for publication June 14th, 1923.

In view of the fact that in man the post-mortem room rarely reveals cancerous metastases in the spleen (although, according to Kettle (1912–13), microscopic nodules are more common in that organ than might be supposed), it was decided to investigate the question in the case of the rat and Jensen's rat sarcoma.

Previous work had shown us that when simultaneous grafts of the same sample of tumour are made into numerous organs of the same animal growth occurs less frequently, and the resulting mass of tumour is smaller in the spleen than in most other tissues. Thus in experiments upon 36 rats, the mean amounts of tumour found at death in the various organs and the frequency of growth in them were as follows:

Mean Amount of Tumour in cubic millimetres.

Sub-cutaneous.	Intra-peritoneal.	Kidney.	Liver.	Testicle.	Spleen.	Brain.
1071	1675	902	604	470	333	22

Percentage Frequency of Definite Growth.

72%	61%	67%	58%	60%	50%	<50%

To test the question, series of rats were inoculated intra-splenically or subcutaneously with portions of a rapidly growing Jensen's rat sarcoma either in the form of fresh tumour or in that of material altered by repeated freezing and thawing or by exposure to a heavy dose of X-rays, and the effect of this procedure upon development of a subcutaneous graft or established tumour was considered. The degree of effect in each series of rats was estimated by (1) the amount of tumour present at the site of the subcutaneous test inoculation on the 10th day, and (2) the ultimate survival of healthy tumour-free rats. Each step of the experiment was associated with numerous controls.

EXPERIMENTS USING FRESH TUMOUR GRAFTS THROUGHOUT.

1. *The Effects of Intra-splenic or Subcutaneous Immunisation upon the Development of a Subcutaneous Graft.*

Twenty normal rats were inoculated into the right flank and 20 into the spleen with grafts of a rapidly growing Jensen's rat sarcoma, and seven days later the survivors received grafts into the left flank.

On the 10th day after the second (test) inoculation the condition in the left flanks of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour. Per rat of series.	Per cent. of control.
Intra-splenic immunisation	5 (30%)	49 c.mm.	26
Subcutaneous immunisation	13 (65%)	103 ,,	56

The numbers of healthy and tumour-free rats at the conclusion of the experiment, 8 weeks after immunisation and 7 weeks after the test subcutaneous inoculation are given in Table I.

The experiment was repeated, and on the 10th day after the second (test) inoculation the condition in the left flanks of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour. Per rat of series.	Per cent. of control.
Intra-splenic immunisation	5 (25%)	35 c.mm.	11
Subcutaneous immunisation	4 (20%)	76 ,,	25

The numbers of healthy and tumour-free rats at the conclusion of the experiment, 5 weeks after the immunising and 4 weeks after the test inoculation are seen in Table I.

TABLE I.—*Numbers of Healthy and Tumour-free Animals at End of Experiment.*

	Intra-splenic immunisation.	Subcutaneous immunisation.
Exp. 1	3 rats (23%)	None.
Exp. 2	14 rats (74%)	2 rats (10%)

On the basis of the observed mortality of the controls after subcutaneous inoculation it was to be expected theoretically that none of the doubly inoculated rats in Exp. 1 (100% and 100% deaths from tumour) would survive, and in Exp. 2 (90% and 70% deaths from tumour) that 3% of them would survive.

From these experiments we conclude that a definite degree of immunity against subcutaneous inoculation is conferred by intra-splenic immunisation with fresh tumour. This immunity is greater than that conferred by subcutaneous immunisation.

2. *The Effects of Intra-splenic or Subcutaneous Inoculation with fresh Tumour Grafts upon an Established Subcutaneous Tumour.*

During the investigations upon which this and an earlier paper (Lazarus-Barlow and Parry, 1923) are based, three series of 20 rats each were inoculated subcutaneously with fresh tumour at a time when a 7 days' tumour was existent in the other flank. The course of the established growth was apparently unaltered. For this reason intra-splenic inoculation alone is considered here.

A number of normal rats received subcutaneous grafts on both sides of the body of a rapidly growing tumour. On the 8th day after inoculation 17 animals with definite subcutaneous nodules received intra-splenic grafts. Other 19 animals bearing tumours were similarly treated on the 13th day after subcutaneous inoculation. Measurements of the subcutaneous nodules in all animals were made at 2-day intervals.

The course of test (subcutaneous) tumour growth after the intra-splenic inoculation in the two series of animals is seen in Table II where are given the mean volumes of the experimental test tumours, expressed as percentages of the controls.

TABLE II.

8-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
119	89	80	78

13-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
80	75	86	88

Ultimately all the control and experimental animals died as the result of enormous tumour growth.

From these experiments it appears that the effects of the intra-splenic inoculation of fresh tumour upon an 8-day and a 13-day established and growing subcutaneous tumour are alike in kind but differ in degree. In both there is an immediate inhibition of rate of growth which causes the experimental tumour to lag behind the control. For an 8-day tumour this lag amounts to about 33%, and is still observable three weeks after the intra-splenic inoculation. For a 13-day tumour the lag is much smaller and lasts for less than a week. No evidence was obtained of a definite retrogression of growth in any instance, though the consistency of the experimental tumours appeared to be less, and deep as well as superficial flattening occurred in some cases.

EXPERIMENTS USING FOR PRELIMINARY INOCULATION GRAFTS OF TUMOUR THAT HAD BEEN REPEATEDLY FROZEN AND THAWED.

Inasmuch as fresh grafts when inoculated into spleen may undergo development into definite tumours (although this is less likely to occur than when a graft is subcutaneous), it was desirable to determine whether an immunity could be produced by the inoculation of material that was altered chemically as little as possible but nevertheless was incapable of multiplication. Previous experiments by Haaland (1910) had shown that living cells are necessary for the production of immunity when inoculation is subcutaneous, but the question is unsolved in respect of intra-splenic inoculation. Various means for attaining this end were tried, notably grinding tumour with sand, killing of cells with chloroform, repeated freezing and thawing. Ultimately

the last-mentioned method was adopted, and since it was found that six times repeated freezing of a thin slice of a 100% (subcutaneous) tumour on the plate of an ether freezing microtome followed by rapid thawing prevented growth of the graft in 80% of the cases, eight times repeated freezing and thawing was used in the experiments to be described.*

(1) *The Effects of Intra-splenic or Subcutaneous Immunisation with Frozen and Thawed Tumour upon the Development of a Subcutaneous Graft of Fresh Tumour.*

Eighteen normal rats received intra-splenic and other twenty received subcutaneous grafts of a tumour which had been frozen and thawed eight times and yielded no trace of growth in any of the controls. Seven days after immunisation all the animals received in the left flank grafts of a fresh rapidly growing tumour.

On the 10th day after the test (subcutaneous) inoculation the condition in the left flank of the rats was as follows:

	Number of rats showing tumours.	Mean volume of tumour. Per rat of series.	Per cent. of control.
Intra-splenic immunisation	11 (65%)	134 c.mm.	55
Subcutaneous immunisation	16 (80%)	219 c.mm.	90

The number of healthy and tumour-free rats at the end of the experiment 5 weeks after the immunising and 4 weeks after the test inoculation are seen in Table III. In spite of the fact that all the subcutaneously-inoculated and 71% of the intra-splenically inoculated rats bore tumours at the end of the experiment, the mean size of these tumours was about half that obtaining in the controls.

TABLE III.—*Numbers of Healthy and Tumour-free Animals at End of Experiment.*

Intra-splenic immunisation.	Subcutaneous immunisation.
4 rats (29%)	None.

We conclude from these experiments that intra-splenic immunisation with repeatedly frozen and thawed tumour confers a certain degree of general resistance to later subcutaneous inoculation with fresh tumour. General resistance induced in this way is greater than that following subcutaneous immunisation. After intra-splenic or subcutaneous immunisation with repeatedly frozen and thawed material a subcutaneous graft of fresh tumour develops at a slower rate than the control.

(2) *The Effects of Intra-Splenic Inoculation of Frozen and Thawed Tumour upon an Established Subcutaneous Tumour.*

Eighteen rats were taken for each of these experiments. The frozen and thawed material used for intra-splenic inoculation in combination with 8-day

* Use of liquid air would be more rapid but was not available; rapid thawing was carried out in the 60° C. paraffin oven.

tumours yielded no trace of growth in any of the controls; that used for intra-splenic inoculation with 13-day tumours yielded small growths after a long delay in 30%.

The course of test (subcutaneous) tumour growth after the intra-splenic inoculation in the two series of animals is seen below in Table IV, where are given the mean volumes of the experimental test tumours expressed as percentages of the controls.

TABLE IV.

8-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
134	86	81	79

13-day Subcutaneous Tumour.

Before intra-splenic inoculation.	1st week after.	2nd week.	3rd week.
77	68	78	78

Ultimately all the control and experimental animals except one of the 8th-day series died as the result of enormous tumour growth. Hence no essential difference was observed between the action of fresh and of repeatedly frozen and thawed tumour material, when injected intra-splenically, upon the growth of an established and growing subcutaneous tumour. In both cases a delay in growth is produced but no retrogression occurs.

EXPERIMENTS USING FOR IMMUNISATION GRAFTS OF TUMOUR THAT HAD BEEN EXPOSED TO A HEAVY DOSE OF X-RAYS.

Chambers, Scott and Russ (1922), practising subcutaneous inoculation in all cases, have shown that protection against a subsequent inoculation is conferred when the material used for the preliminary grafts has been subjected to a "lethal" dose of X-rays under standard conditions. It appeared desirable to apply the method in the case of intra-splenic inoculations also. Batches of 20 normal rats were therefore inoculated intra-splenically with Jensen's rat sarcoma that had been exposed for 55 minutes and 66 minutes respectively to X-rays under standard conditions.* Subcutaneous controls and intra-splenic and subcutaneous controls with non-radiated material were made at the same time. A week later all the rats were inoculated subcutaneously with grafts of a rapidly-growing tumour.

So far as concerns the effects of subcutaneous immunisation with heavily-irradiated material we confirm the observations of Chambers, Scott and Russ, for 42% of the rats immunised with 55 minutes' irradiated material and 60% of those immunised with 66 minutes' irradiated material developed no growth, whereas over 97% of the controls died from tumour.

In the case of intra-splenic immunisation with irradiated material the

* The exposures were kindly carried out for us by Miss Gladwys Scott.

result of our experiments was not so clearly defined. Under these conditions a larger proportion of the animals developed growths at the site of test subcutaneous inoculation than was the case when non-radiated material was used for immunisation. There was evidence also from the volume of the subcutaneous tumour on the 10th day after test-inoculation that growth was more rapid when the material used for intra-splenic immunisation had been irradiated. Thus the mean volume of the test tumour on the 10th day after inoculation was, in the 55 minutes' series, 3 times, and in the 66 minutes' series nearly 6 times that which obtained in the corresponding series in which the immunising grafts had not been exposed to X-rays. At the end of the experiment, in the 55 minutes' series 40% of the rats (controls 0) and in the 66 minutes' series 42% (controls 25%) were healthy and tumour-free, while in the corresponding series in which the immunising grafts had not been irradiated the percentages of healthy and tumour-free animals were 23% (controls 0) and 74% (controls 5%) respectively.

These results must not be taken to imply that no resistance is produced by intra-splenic immunisation with irradiated tumour, for all the controls that were subcutaneously inoculated with non-radiated tumour died of growth. It appears to indicate, however, that with intra-splenic immunisation a smaller degree of initial and no greater degree of terminal general resistance to a later subcutaneous test inoculation is conferred when that immunisation is made with heavily radiated than when it is made with non-radiated material.

In this connection it is noteworthy that for no readily assignable reason deaths of the animals soon after intra-splenic inoculation with irradiated material were, in our experience, markedly more numerous than when non-radiated material was used intra-splenically, or radiated or non-radiated material was inoculated subcutaneously or intra-cerebrally. In a total of 348 rats operated upon by the same one of us (R. H. P.), the deaths for no readily-assignable reason during the first seventeen days after the first inoculation were as follows: Subcutaneous inoculations—non-radiated material 2%, irradiated 3%; intra-cerebral inoculation—non-radiated material 6%, irradiated 8%; intra-splenic inoculation—non-radiated material 8%, irradiated 31%.

COMPARISON BETWEEN THE EFFECTS OF INTRA-SPLENIC, INTRA-CEREBRAL AND SUBCUTANEOUS INOCULATION.

In another place, we, Lazarus-Barlow and Parry (1923), have brought forward evidence to show (1) that an intra-cerebral graft meets with a degree of local resistance to its growth which does not obtain in the case of a subcutaneous graft, and (2) that a greater degree of general resistance to later subcutaneous inoculation follows intra-cerebral immunisation than follows one beneath the skin. While the experiments that form the subject of the present paper were being carried out, intra-cerebral inoculations were made on series of 10 rats for the purpose of comparison. These confirmed our previous conclusions, and in addition gave evidence of a greater degree of *local* resistance in the spleen than in the brain, and of a greater degree of *general* resistance to a later subcutaneous inoculation after an intra-splenic than after an intra-cerebral immunisation.

The evidence for a greater *local* resistance in spleen than in brain consists in the fact that whereas in Exp. 1* 100% and in Exp. 2, 83% of intra-cerebral grafts developed tumours, only 59% and 21% of intra-splenic grafts followed this course.

The contrast between local resistance in spleen and in subcutaneous tissue is even more striking, as is shown in Table V.

TABLE V.

Condition of graft.	"Takes" per cent. of cases. Intra-splenic.	Subcutaneous.
Fresh	59	100
,,	21	90
,,	77(1)	95
Irradiated	0	26(2)
,,	0	10(2)
Frozen and thawed	0	30(2)

(1) Subcutaneous tumour present when inoculated.
(2) Four weeks' delay in appearance of growths.

The evidence for a greater degree of *general* resistance to a later subcutaneous inoculation, conferred by intra-splenic as contrasted with intra-cerebral immunisation, is afforded by the fact that in Exp. 1, none, and in Exp. 2, 10% of the rats that had received intra-cerebral grafts were healthy and tumour-free at the conclusion of the experiment, whereas of those which had received intra-splenic immunisation 23% and 74% respectively were healthy and tumour-free. When heavily irradiated material was used for the intra-cerebral or intra-splenic immunisation a similar contrast between the two organs was observed.

Using the same criterion and combining all the available experiments detailed in this paper, 22 out of 95 rats (23%) that had received subcutaneous immunisation were healthy and tumour-free at the end of the experiment, whereas for those which had received intra-splenic immunisation the numbers were 30 out of 68 (44%). Eliminating experiments in which irradiated tumour was used for immunisation, the value for subcutaneous immunisation was 4% and for intra-splenic immunisation 46%.

THE DEGREE OF GENERAL RESISTANCE, WHEN PRESENT, CONFERRED BY A PRELIMINARY INOCULATION.

In the preceding sections frequently it has been stated that animals were "healthy and tumour-free" at the end of the experiment. In many instances this fact could only be determined after killing the animal, but in others the deduction was made from the general condition of the rat. Rats of the latter category were subjected to a third inoculation (subcutaneous) with an actively growing tumour to determine the degree of immunity that was present. 51 of such animals were re-inoculated at periods varying from two weeks to two

* The local resistance presented by brain was, in this experiment, manifested by delay in growth alone and not by failure of the graft to take in addition to delay. Inoculated subcutaneously the tumour mortality was 100%.

months after a second inoculation. In 48 of these the graft did not develop at all, or developed for a short time and subsequently disappeared. In the remaining 3 a small, slowly-growing tumour appeared beneath the skin, was present when the animal was killed, and presented normal histological features. The immunity induced is, therefore, of a high order.

HISTOLOGY OF SPLEEN.

Routine histological examination was made of the spleen, and in 59 cases glycerine films of the pulp were prepared for cell examination. No very definite results accrued from this work. The most that can be said is that when fresh grafts had been inoculated into spleen the splenic cells were larger and there was a larger number of pyronin (plasma) cells under Pappenheim's stain than when irradiated material had been used. The occurrence of these pyronin-staining cells appears to be associated with the presence of actual tumour growth in the spleen, for there was found on an average 1 such cell to 12 other splenic cells when there was a complete absence of growth in the body, or when growth was present elsewhere in the body but absent from the spleen, whereas the ratio was 1 : 7 when the spleen itself was the seat of growth. In this connection it is noteworthy that the cytoplasm of Jensen's rat sarcoma cells has a marked avidity for pyronin. No correlation between the number of pyronin-staining cells and the degree of general immunity was detected and no unusual types of cell were found. Whether fresh or irradiated material was used as graft the number of typical lymphocytes in the spleen appeared to be unusually small. Though definite measurements are not available, the spleen was certainly much larger in cases of intra-splenic than in cases of intra-cerebral inoculation, even though the intra-splenic graft itself failed to develop. Indeed the organ appeared to be larger than normal with intra-splenic and smaller than normal with intra-cerebral inoculation.

CONCLUSIONS.

We conclude in the case of the rat and Jensen's rat sarcoma that :

(1) The spleen offers a greater local resistance to growth of a tumour graft than brain or subcutaneous tissue.

(2) After intra-splenic immunisation the general resistance to later subcutaneous inoculation is raised, and that to a greater extent than after intra-cerebral or subcutaneous immunisation.

(3) Intra-splenic inoculation retards growth in an established subcutaneous tumour but does not lead to its disappearance.

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SPONTANEOUS DESENSITISATION.*

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Received for publication June 21st, 1923.

THE early experiments of Rosenau and Anderson (1909), who found that guinea-pigs two and even three years after the injection of horse-serum were still extremely sensitive to the intravenous injection of the antigen, led them to the view, which is still generally held, that anyphylactic sensitiveness produced by a single injection of alien protein persists throughout the life of the animal. In some experiments on immune guinea-pigs Dale (1913) noted that the plain muscle, three months after the last immunising injection, had largely lost its sensitiveness. More recently Thomsen (1917) has shown quantitatively that actively anaphylactic guinea-pigs undergo a definite diminution of sensitiveness with the lapse of time—a change which depends only in part upon the protection against the injection of antigen which is afforded them by their increasing age and weight. Weil (1913) demonstrated the difference which exists between guinea-pigs sensitised passively with homologous and those sensitised with heterologous immune serum. The former were still sensitive on the 70th day, while in the latter sensitiveness to the antigen disappeared in a few days, and was replaced by active sensitiveness towards the heterologous serum proteins.

Our experiments concern (1) the influence of partial immunisation produced by very small doses of antigen in actively sensitive guinea-pigs upon the rate at which spontaneous desensitisation occurs, and (2) the rate of spontaneous desensitisation in guinea-pigs passively sensitised with homologous immune serum.

SPONTANEOUS DESENSITISATION IN ACTIVELY SENSITIVE GUINEA-PIGS.

A large series of guinea-pigs were actively sensitised by the subcutaneous injection of 1 mg. of crystalline horse-serum albumin. Some of these were subjected to no further treatment, and the tests of their sensitiveness one month and twelve months after the sensitising injection gave us information as to the extent to which spontaneous desensitisation occurred in this series of actively sensitive animals, and at the same time served as a control for the further experiment concerning the influence of immunisation. One month after the sensitising dose tests by the intravenous injection of antigen gave the following results:

* The experiments described in this paper were carried out while Kellaway was Foulerton Student of the Royal Society, and their cost was defrayed by a grant from the Graham Research Fund.

Wt. of G.P.	Dose of antigen.	Result.
265 gm.	0·1 mg.	Slight symptoms, recovery.
255 gm.	0·2 mg.	† in 3½ minutes.
330 gm.	0·2 mg.	† in 3 minutes.

M.L.D. = 0·2 mg.

One year after the sensitising injection the animals were much larger and their sensitiveness had greatly diminished, as the following results show:

Wt. of G.P.	Dose of antigen.	Result.
550 gm.	0·5 mg.	Moderate symptoms, recovery.
540 gm.	2·0 mg.	Severe symptoms, recovery.
500 gm.	5·0 mg.	,, ,, ,,
600 gm.	20·0 mg.	† in 5¼ minutes.

A second dose of 10 mg. of antigen ten minutes after the first injection failed to cause any symptoms in the first two animals in this table. The M.L.D. lies somewhere between 5 and 20 mg., and is probably about twenty times as great per kilo of body-weight as it was eleven months before. During this period the animals had doubled their weight, and the large size of their bronchial tubes doubtless helped to protect them from any but large doses of antigen.

In Thomsen's experiments, in which horse-serum was used as the antigen, a similar degree of spontaneous desensitisation occurred in the course of a year. By testing the sensitiveness of the plain muscle by Dale's (1913) method we were able to avoid the complicating effect of the increase in size of the animals, and to make our results quantitative without the use of a very large number of guinea-pigs. Two months after the sensitising injection the plain muscle gave a small reaction to a concentration of 1 in 1,200,000 of the antigen, but reacted maximally to 1 in 240,000. Ten months later a concentration of 1 in 12,000 of the antigen was required to produce a maximal response and only a small reaction occurred to 1 in 240,000.

Spontaneous desensitisation in guinea-pigs is, therefore, a very slow process. We have not followed the change beyond the first year after the acquirement of sensitiveness and are unable to say whether it is ever complete.

THE INFLUENCE OF PARTIAL IMMUNISATION ON SPONTANEOUS DESENSITISATION IN ACTIVE ANAPHYLAXIS.

A number of the anaphylactic guinea-pigs of this series one month after the sensitising dose were temporarily desensitised by the subcutaneous injection of 1 mg. of crystalline horse-serum albumin. They were then partially immunised by an intravenous injection of 0·5 mg., followed by a series of five subcutaneous injections of 1 mg. of horse-serum albumin at weekly intervals. One or more of these animals were killed at various intervals after the last immunising injection, and their plain muscle was perfused and tested. The results are given in the following table. It should be noted that the animals tested one month after immunisation received only two immunising injections.

Time in relation to immunisation.	Dose of antigen (horse-serum albumin).	Reaction of plain muscle.
Before	0·5 mg.	Maximal.
	0·1 mg.	Small.
1 month after	0·5 mg.	Maximal.
	0·1 mg.	Maximal.
	0·02 mg.	Small response.
2 months after	0·5 mg.	Maximal.
	0·1 mg.	Large, but poorly sustained response.
4 months after	1·0 mg.	Small, modified response.
	10·0 mg.	Large, but poorly sustained response.
6 months after	1·0 mg.	Very small response.
	10·0 mg.	Maximal.
8 months after	10·0 mg.	Moderate poorly sustained response.
	20·0 mg.	Maximal in extent, poorly sustained.
9 months after	10·0 mg.	*Nil.*
	20·0 mg.	*Nil.*
	30·0 mg.	Very small.

The extent of the anaphylactic response of the plain muscle was estimated in all cases by comparison with the contraction obtained by the subsequent addition of 0·05 mg. or 0·1 mg. of histamine to the bath, which contained 120 c.c. of oxygenated Ringer's fluid. The decrease in the sensitiveness of the plain muscle of these partially immunised guinea-pigs is more marked than that which occurs in untreated actively anaphylactic animals of the same series. Whereas one month after partial immunisation a maximal reaction was obtained to a concentration of 1 in 1,200,000, nine months after the last immunising injection the plain muscle gave only a minute response to a concentration of 1 in 4000 of the antigen. At this time, a year after sensitisation, the plain muscle of untreated actively sensitive guinea-pigs of this series reacted maximally to a concentration of 1 in 12,000 of the antigen, so that even the very partial immunisation conferred by the injection of small doses of antigen was effective in accelerating the normal slow rate of spontaneous desensitisation.

The extent to which these animals were immunised was shown by the capacity of their sera to confer passive sensitiveness. 2 c.c. of the serum from an animal two months after the last immunising injection, administered intra-peritoneally to a normal guinea-pig weighing 250 gm., produced in 48 hours such a degree of sensitiveness that the plain muscle reacted maximally to a concentration of 1 in 60,000 and almost maximally to 1 in 120,000 of horse-serum albumin. The plain muscle of a normal guinea-pig which had

been injected 48 hours before with 5 c.c. of this serum reacted maximally to a concentration of 1 in 240,000, and gave a large response to 1 in 1,200,000 of the antigen. 5 c.c. of the serum from an animal four months after immunisation contained much less antibody, since the plain muscle of the passively sensitive guinea-pig injected with this dose of serum gave only a moderate response to a concentration of 1 in 40,000 of the antigen. At later periods after immunisation we were unable to demonstrate any antibody in the serum by passive sensitisation experiments.

In the course of these experiments we made some observations on the duration of specific desensitisation after a single subcutaneous injection of 1 mg. of antigen. Seven male guinea-pigs of the same series 30 days after the sensitising injection were specifically desensitised in this way. They were tested by the intravenous injection of antigen at various periods with the following results:

Weight of G.P.	Days after densensitisation.	Dose of antigen.	Results.
295 gm.	2	0·2 mg.	Slight symptoms; recovery.
270 gm.	5	0·2 mg.	*Nil.*
250 gm.	8	0·2 mg.	Moderate symptoms; recovery.
350 gm.	9	0·2 mg.	Slight symptoms; recovery.
325 gm.	9	0·5 mg.	Very severe symptoms; only just recovered.
280 gm.	12	0·2 mg.	Slight symptoms; recovery.
260 gm.	12	0·5 mg.	Very severe symptoms; recovery after 2 hours.

The temporary and partial protection afforded by the subcutaneous injection of small doses of antigen lasted longer than we had anticipated. After 12 days there was still some protection to $2\frac{1}{2}$ M.L.D. of the antigen.

Our experiments suggest that the method of protecting man from possible anaphylactic shock by repeated *small* subcutaneous injections of the alien protein to which he is sensitive, in addition to affording temporary and partial protection by specific desensitisation, has the advantage of greatly increasing the rate at which spontaneous desensitisation normally occurs.

SPONTANEOUS DESENSITISATION IN GUINEA-PIGS PASSIVELY SENSITISED WITH HOMOLOGOUS SERUM.

Weil (1914) showed that antigen as well as antibody was present in the serum of guinea-pigs immunised against a foreign protein, and it seemed possible that persistence of sensitiveness in animals passively sensitised with such serum 70 days after the sensitising injection might be due to the development of active sensitiveness in response to traces of antigen in the serum. We attempted to discover if this were the case by testing, at various periods after sensitisation, the plain muscle of a series of guinea-pigs passively sensitised with immune guinea-pig serum. The immune serum used in these experiments was obtained by injecting a series of four intra-peritoneal and

four subcutaneous doses of 0·5 c.c. of horse-serum into guinea-pigs at weekly intervals. Thirteen days after the last injection several of these animals were bled out and the pooled serum was titrated by testing its power to confer sensitiveness on normal guinea-pigs.

Wt. of G.P. in gms.	Dose of serum intraperitoneally.	Result of intravenous injection of 0·1 c.c. of horse-serum after 48 hours.
240	1·0 c.c.	† in 5½ minutes.
250	1·0 c.c.	† in 5¾ minutes.
240	0·5 c.c.	Very severe symptoms; just recovered.
240	0·5 c.c.	† in 4¼ minutes.
200	0·2 c.c.	*Nil.*

The minimal sensitising dose of this pooled serum (against a dose of 0·1 c.c. of horse-serum) was between 0·5 c.c. and 1·0 c.c.

A series of twelve female guinea-pigs were now sensitised by the intraperitoneal injection of 1 c.c. of this immune serum and their plain muscle was perfused and tested at various periods after injection, with the following results:

Time after injection of immune serum.	Dose of antigen (horse-serum).	Reaction of the plain muscle.
1 day	0·02 c.c.	½ maximal.
	0·1 c.c.	¾ maximal, poorly sustained.
2 days	0·01 c.c.	½ maximal.
	0·1 c.c.	¾ maximal.
4 days	0·01 c.c.	Nearly maximal.
	0·1 c.c.	Maximal.
6 days	0·004 c.c.	¾ maximal.
	0·01 c.c.	Nearly maximal.
9 days	0·01 c.c.	*Nil.*
	0·1 c.c.	Small response.
9 days	0·1 c.c.	¾ maximal.
	0·5 c.c.	Maximal.
11 days	0·01 c.c.	¾ maximal.
	0·5 c.c.	Maximal.
14 days	0·01 c.c.	*Nil.*
	0·5 c.c.	Nearly maximal.
16 days	0·5 c.c.	Maximal.
	0·01 c.c.	Very small.
23 days	0·01 c.c.	Very small response.
	0·5 c.c.	½ maximal.
31 days	0·01 c.c.	*Nil.*
	0·1 c.c.	*Nil.*
31 days	0·1 c.c.	*Nil.*
	1·0 c.c.	*Nil.*

The maximal sensitiveness which we detected was at the 4th and 6th days, when a concentration of 1 in 30,000 of horse-serum gave a ¾ maximal response, while the reaction to 1 in 12,000 was almost maximal. After this time there was a fall in sensitiveness, so that on the 14th day similar responses were only obtained to a concentration of 1 in 240 of horse-serum. By the 31st day the plain muscle was not sensitive to a concentration of 1 in 120 of the antigen. Stronger concentrations could not be tested, since in these horse-serum has a stimulant effect on plain muscle apart from its antigenic property. The reaction of the whole animal to the intravenous injection of horse-serum gave confirmatory results. Two days after the sensitising injection 0·1 c.c. of horse-serum invariably killed animals sensitised with 1·0 c.c. of this immune serum, whereas in 4 guinea-pigs tested 31 days after sensitisation the injection of 5·0 c.c. of horse-serum produced no anaphylactic symptoms.

Diminution of sensitiveness was not, within the time of our experiment, followed by an increase of sensitiveness, as might have been the case if passive were replaced by active sensitiveness. Spontaneous desensitisation occurred at a much more rapid rate than in actively anaphylactic animals.

There seems to be a definite tendency to spontaneous desensitisation in all forms of anaphylactic sensitiveness in the guinea-pig. The rate of desensitisation is most rapid in passive sensitiveness with heterologous serum, occurring in a few days (Weil). In animals passively sensitised with homologous serum, sensitiveness in our experiments was not demonstrable after 30 days. In actively anaphylactic guinea-pigs there is a marked decline in sensitiveness during the first year after sensitisation, and this slow spontaneous desensitisation is greatly accelerated by immunisation.

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THE ACID-BASE BALANCE IN THE PLASMA IN THE LATER STAGES OF PREGNANCY.

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Received for publication July 9th, 1923.

HASSELBALCH and Gammeltoft (1915) found evidence that the blood was less alkaline during the later stages of pregnancy than after delivery. Losee and Van Slyke (1917) also found that the plasma bicarbonate during pregnancy was below normal. These results indicate that there is in pregnancy an accumulation of acid in the blood or depletion of alkali, although Losee and Van Slyke's results are inconclusive, as they did not allow for the possibility of reduction of plasma bicarbonate produced by reduction of CO_2 tension by hyperpnœa.

Low values for plasma bicarbonate may be due to accumulation of acid in or loss of base from the blood, causing a relative excess of acid which combines with some of the base usually present as bicarbonate; or due to migration of Cl-ions out of the red blood-corpuscles in response to reduction of the CO_2 tension of the blood, in which case the base of the usual bicarbonate is used up to combine with these Cl-ions, although there is no excess of acid in the blood as a whole.

These two conditions may be distinguished by determining also the CO_2 tension or the hydrogen-ion concentration of the plasma. It is convenient to use a diagram as in Fig. 1; in this ordinates represent concentration of bicarbonate in plasma in gramme equivalents per litre, abscissæ CO_2 tension in mm. of mercury. A point representing a plasma is fixed if the plasma CO_2 tension (abscissa) and bicarbonate (ordinate) are known. Since the H-ion concentration is proportional to the ratio CO_2 tension : plasma-bicarbonate, points representing plasmata with the same H-ion concentration lie on straight lines passing through the origin; such lines are shown in the diagram, calculated according to the formula given by Austin *et al.* (1922), and designated in terms of pH. The point representing a plasma can also therefore be fixed if the bicarbonate (giving the ordinate) and pH (giving the diagonal line on which the point lies) are known.

If samples of plasma are separated from the same blood at varying CO_2 tensions, points representing these samples of plasma will lie on a curve such as A_1, A_2 (which may be called a true plasma dissociation curve). If from the same person samples of blood are taken (1) after normal breathing when the CO_2 tension is about 50 mm., and (2) after excessive lung ventilation when the venous CO_2 tension is 30 mm., the bicarbonate content of plasma separated from these two samples of blood will be 0·0275 and 0·0225 gm. equivalents per litre. But if we know the pH of these plasma samples we can fix the points representing them, and, finding that they lie on the same curve, conclude that the reduction of plasma bicarbonate is due only to reduction of

CO_2 tension. If, on the other hand, non-volatile acid accumulates in the person's blood, the plasma bicarbonate at a given CO_2 tension will be lower, and the dissociation curve will be lower than before; this is illustrated by the points K_1 and K_2, representing the plasmata of two patients with moderate and severe ketosis (0·094 and 0·305 gm. of acetone bodies as β-hydroxybutyric acid per litre).

Methods.—The subjects of this investigation were pregnant women attending the antenatal clinic of the London Hospital; no pathological changes had been found on clinical examination. The blood was taken

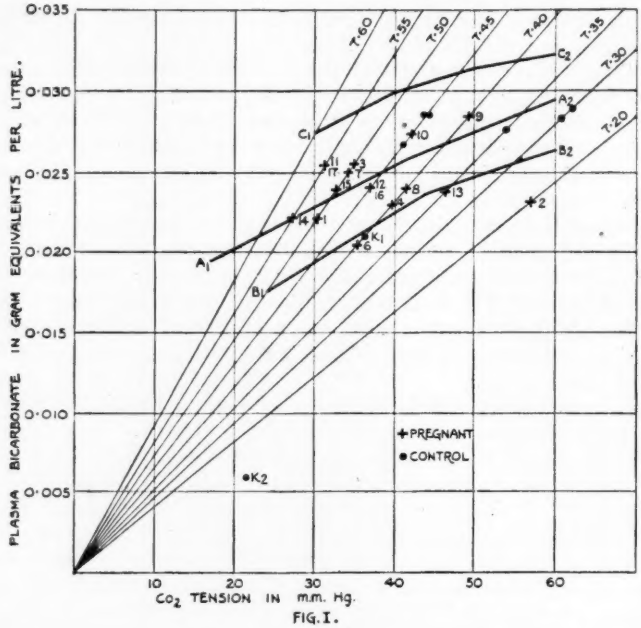

FIG. I.

between 11.45 a.m. and 12.15 p.m. and the plasma separated without loss of CO_2 as described by one of us (Marrack, 1923). The plasma so separated was used for all the estimations.

Hydrogen-ion concentration and plasma bicarbonate were determined by the methods of Cullen (1922) and Van Slyke (1922): chlorides, by boiling with excess of silver nitrate in the presence of nitric acid and titrating the excess of silver nitrate with potassium thiocyanate; sodium, by the direct method of Kramer and Tisdall (1921), checked by the incineration method of Kramer (1920). It was found essential to separate the plasma at once from the corpuscles, to avoid low values owing to diffusion of sodium into the corpuscles.

As controls six young women students and graduates of this hospital were bled shortly before lunch, at times from 11.30 a.m. to 12.30 p.m. It must be

remembered in interpreting the results that these controls were of exceptionally good physique and therefore not strictly comparable with the hospital out-patients, although these also were above the standard of ordinary hospital patients.

The true plasma dissociation curves given by normal bloods lie between B_1, B_2 and C_1, C_2; this range is calculated from the whole blood dissociation curves given by Peters, Barr and Rule (1921). The six controls come well within these boundaries, and lie very close to each other so far as height of curve is concerned. As may be expected in venous blood, the CO_2 tension and pH vary.

TABLE I.

No.	Number of weeks pregnant	pH	HCO_3^-	Cl^-	$HCO_3^- + Cl^-$	Na^+	Acetone bodies	Urine
			Gram equivalents per litre					
1	36	7·50	0·022	0·106	0·128	0·135	0·001	
2	36	7·25	0·023	0·1025	0·1255	0·133	0·0015	Haze of albumen. Acetone.
3	37	7·50	0·0255	0·1045	0·130	0·1305	0·001	Trace of albumen. Acetone.
4	38	7·40	0·023	0·106	0·129	0·1305		
5	38	–	0·023	0·100	0·123	0·1305		Trace of albumen.
6	32	7·40	0·0205	–	–	–		
7	32	7·50	0·025	0·100	0·125	0·1305	0·001	
8	34	7·40	0·024	0·101	0·125	0·1305	0·001	Acetone.
9	32	7·40	0·0285	0·095	0·1235	0·135		
10	30	7·45	0·0275	–	–	0·128		Haze of albumen. Few granular casts.
11	35	7·55	0·0255	0·106	0·1315	–		
12	36	7·45	0·024	0·1015	0·1255	–		
13	32	7·35	0·024	0·0975	0·1215	0·1305		Glucose present. Blood sugar 0·10 per cent.
14	22	7·55	0·022	0·101	0·123	0·1305		
15	38	7·50	0·024	0·1005	0·1245	0·1305		
16	36	7·45	0·024	0·103	0·127	0·135		Haze of albumen. Few granular casts.
17	32	7·55	0·0255	0·100	0·1255	0·129		
Average of cases in which HCO_3^- Cl^- and Na^+ were determined			0·0240	0·1009	0·1249	0·1321		

The points representing the plasmata of eleven of the sixteen pregnant women lie on curves of the same level as the controls*; of the other five, only two lie below the normal boundaries. But the pH was distinctly higher and the CO_2 tension lower than in the controls (average CO_2 tension of controls 52·1 mm., of pregnant women 38·3 mm.). This agrees with the low alveolar air CO_2 tensions found by Rowe (1923) in pregnant women. The values found for plasma bicarbonate are rather low, 0·024 gm. equivalents per litre instead of 0·0277, or, using the old method of expression, 54 c.c. of CO_2 per 100 c.c. of plasma instead 62 c.c. This is as it should be in plasmata derived from blood at low CO_2 tensions.

* The inference of the height of the dissociation curve from one point assumes that the curves of the cases under consideration do not differ notably in slope from normal; appreciable deviation from normal is only likely to occur in severe anæmia, which was not present in any of our patients. The curves found by Hasselbalch and Gammeltoft (1915) in pregnancy, determined by two points, were of normal slope.

Acetone bodies were present in the urines of some of the subjects, but in those cases in which the acetone bodies in the blood were estimated, the excess found above normal (about 0·0005 gm. equivalents per litre) was not enough to cause appreciable reduction of plasma bicarbonate.

In Tables I and II are given the concentrations of HCO_3, Cl and Na-ions in these plasmata. From these data the balance of anions and kations in the plasmata of pregnant women and controls may be compared. In pregnant women as in other people the concentrations of HPO_4, K, Ca, and Mg-ions (Krebs and Briggs, 1923) vary little from 0·0016, 0·005, 0·005 and 0·0025 gm. equivalent per litre. The sum of all the kations in the plasma can be obtained from the figures for Na by adding 0·0125. This gives in the controls an excess of kations unaccounted for by HCO_3, Cl, and HPO_4-ions, equivalent to 0·019: most of this is combined with protein; in the pregnant women the excess is 0·018. These figures probably agree within the limits of error in Na-ion estimations, but Dr. Coetzee found in these cases that the plasma protein of the pregnant women was less than that of the controls (7·39 instead of 8·05 per cent.); so that a difference of the order found in the amounts of kation combined with protein, and therefore of the amounts of kation unaccounted for by HCO_3, Cl, and HOP_4-ions, is to be expected. If appreciable amounts of abnormal acid were present in the plasmata of pregnant women this should reveal itself by an abnormal excess of kations; this we have not found.

TABLE II.

pH	HCO_3^-	Cl^-	$HCO_3^- + Cl^-$	Na^+
	GRAM EQUIVALENTS PER LITRE.			
7·35	0·0275	0·100	0·1275	0·135
7·45	0·0265	0·104	0·1305	0·139
7·45	0·0285	0·100	0·1285	0·137
7·45	0·0285	0·1006	0·129	0·141
7·30	0·0290	0·098	0·127	0·135
7·30	0·0285	0·104	0·1325	0·141
AVERAGE	0·0277	0·102	0·1297	0·138

Our results differ most from those of Hasselbalch and Gammeltoft in that we found as a rule an alkalæmia; this difference is reflected in the urines of the patients; those which we collected corresponding to the period in which the blood was taken were neutral or alkaline, while those of Hasselbalch and Gammeltoft's patients were strongly acid. The lowering of the dissociation curve which they found is not great, as in only three out of eight pregnant cases is the pH at 40 mm. CO_2 more than 0·01 (a difference we should not detect) below that of the blood of Hasselbalch himself.

Such lowering of the dissociation curve as we have found appears to be due to deficiency of alkali rather than accumulation of abnormal acids in the blood.

CONCLUSION.

In the latter half of pregnancy there is as a rule a reduction of CO_2 tension in the blood; this produces a moderate alkalæmia and reduction of plasma bicarbonate. In a minority of cases there is an actual alkali deficit.

We owe our thanks to the physicians of the Ante-Natal Clinic of the London Hospital for the facilities they have afforded for this investigation.

The expenses of part of this work were defrayed by a grant from the Medical Research Council.

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THE CLASSIFICATION OF HÆMOGLOBINOPHILIC BACTERIA, BASED UPON THEIR RELATION TO BLOOD-PIGMENT AND TO THE "VITAMINE" FACTOR.*

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Received for publication July 31st, 1923.

IN their attempt to classify micro-organisms more closely upon a botanical-biological basis the Society of American Bacteriologists (1920) have coined a new genus, "Hemophilus," which provisionally includes such diverse bacteria as *B. influenzæ*, *B. pertussis*, *B. duplex*, Ducrey's bacillus and the Koch-Weeks' bacillus. These bacteria are described as "minute rod-shaped cells . . . growing best (or only) in the presence of hæmoglobin, and in general requiring blood-serum or ascitic fluid."

In this genus, therefore, are included not only the "hæmoglobinophilic" bacteria, but also others which merely have a preference for *blood* in the medium or even for serum or for ascitic fluid.

The biological aspect of this classification does not take into account the leading character of a hæmoglobinophilic bacterium, typified by *B. influenzæ*, namely its dependence for growth upon an iron-containing blood-pigment as pointed out by Pfeiffer in his original paper. As is well known, neither *B. pertussis* nor *B. duplex* are dependent upon blood-pigment.

* Work carried out under a grant from the Medical Research Council.

In more recent years the conditions of growth of *B. influenzæ* have been more closely established. It has been confirmed that blood-pigment is necessary (or according to some, an unspecified heat-stable factor in blood), but it is also found that a heat-labile factor, usually known as the "V" factor, is equally necessary (Davis, 1921; Fildes, 1921; Rivers and Poole, 1921; Thjötta and Avery, 1921[1]).

These characters of *B. influenzæ* are so sharply defined that it appears undesirable to include in the same group as this bacillus other bacteria which have no such dependence upon special food factors, but merely a preference for blood or ascitic fluid when first isolated from the body.

THE RELATION TO GROWTH FACTORS OF CERTAIN SO-CALLED "HEMOPHILIC" BACTERIA.

The degree of dependence upon hæmatin and V factor exhibited by some of the "hemophilic" bacteria and by *B. hæmoglobinophilus canis*, not referred to by the American Committee, was determined as follows:

Eight strains of *B. influenzæ*, obtained from the lungs of cases of pneumonia *post mortem*, were compared with 23 strains of Koch-Weeks' bacillus from patients with muco-purulent conjunctivitis; with 5 recently isolated strains of *B. duplex*; with 4 strains of *B. pertussis* obtained from the National Collection of Type Cultures, and with one strain of *B. hæmoglobinophilus canis* sent to me by Dr. Martin Kristensen, of Copenhagen (Nat. Coll. Type Cultures, No. 1659).

Experiment 1.—The relation of various bacteria to growth factors.

For testing each strain 4 tubes of 1½ per cent. bacto-peptone water were taken. These contained no V factor. To two of the four tubes 0·1 c.c. of a 1 per cent. solution of hæmatin in water was added. This hæmatin was prepared from pure crystallised horse hæmoglobin (for which I have to thank Prof. H. E. Roaf) by drying with anhydrous potassium carbonate, extraction with alcohol and solution of the extract in water. To one of the tubes containing hæmatin and to another peptone water tube 1 c.c. of yeast extract was added. This yeast extract was made according to the directions of Thjötta and Avery (1921[1]), but filtered through a Berkefeld N candle to remove traces of X factor which are adsorbed to suspended particles of yeast.

These 4 tubes were then inoculated with one drop of a very fine suspension of the strain to be tested. The success of all experiments upon growth requirements of hæmoglobinophilic bacteria depends upon absence of hæmatin from the inoculum. The inoculation must obviously be made from a medium containing blood-pigment, but (*a*) the amount of blood in the medium should be as small as possible, viz. 1 per cent. of Fildes' (1920) peptic digest in agar, and (*b*) the culture should be young to reduce absorption of hæmatin upon the bacteria to a minimum, while (*c*) the quantity transferred should be minute, viz. the point of a needle (not loop) is touched upon a slope culture and agitated in 10 c.c. of salt solution; of this one drop is inoculated into each tube of peptone water.

The growth was allowed to continue for two days, or four days in the case of *B. pertussis*, and then subcultures of one loopful were transferred to Fildes'

peptic blood agar, or to ordinary blood agar for *B. pertussis*, to determine the nature of the growth or the absence of growth.

The results with all strains of *B. influenzæ* and Koch-Weeks' bacillus were the same, while those with *B. hæmoglobinophilus canis* and the other bacteria were different. Two of the strains of *B. pertussis* would not grow under these conditions and were excluded.

	B. influenzæ.	Koch-Weeks.	*B. hæmoglobinophilus canis.*	*B. pertussis*, No. 760.	*B. pertussis*, No. 364.	*B. duplex* (3 strains).	*B. duplex* (2 strains).
Peptone-water + 0 . .	0	0	0	+	+	++	++
,, + hæmatin .	0	0	++	+	++	++	0
,, + yeast .	0	0	0	0	++	++	+
,, + hæmatin + yeast	++	++	++	0	++	++	++

++ = Visible growth in the peptone-water tubes and on subculture. + = No visible growth, but growth on subculture. 0 = No growth.

From this experiment it is clear that Koch-Weeks' bacillus has identical growth requirements to *B. influenzæ*, and may thus be associated with it in a classification based upon this character, while *B. hæmoglobinophilus canis* demands hæmatin but is indifferent to the V factor. It may be remarked that none of these strains were hæmolytic, while two of the influenza strains and one of the Koch-Weeks strains produced indol.

B. pertussis and *B. duplex* were in no way dependent for growth upon the factors which are essential for the influenza bacillus.

These results confirm those of Rivers (1922[1]). He tested the growth requirements of *B. influenzæ*, *B. pertussis* and *B. hæmoglobinophilus canis*, using peptone agar as the basal medium.

The demonstrated dependence of the hæmoglobinophilic organisms upon the iron-containing pigment hæmatin does not imply that other substances are not capable of exerting the same effect. Thjötta and Avery (1921[2]) have proved that potato can replace the hæmatin, which, according to Fildes (1922), is due to the fact that the actions of hæmatin and potato are similar. Again, it is known that a trivial growth of *B. influenzæ* may be obtained in impure culture with certain bacteria in the absence of hæmatin (Williams and Povitzky, 1921). This effect is also no doubt due to the peroxidase demonstrable in such bacterial cells by means of guaiaconic acid.

THE SIGNIFICANCE OF THE V FACTOR IN THE GROWTH OF THE HÆMOGLOBINOPHILIC BACTERIA.

The question whether the lack of dependence of *B. hæmoglobinophilus canis* upon the V factor is sufficient to exclude it from classification with *B. influenzæ* is difficult to decide in the absence of exact knowledge as to the identity of the V factor.

The V factor is not confined to the red blood-corpuscles, but is present in extracts from cells of the whole animal and vegetable kingdoms. It is probable,

as has been suggested by Davis (1921) and Rivers and Poole (1921), that it is the V factor which is responsible for the well-known "symbiosis phenomenon" in the growth of the influenza bacillus. This phenomenon is not observed when excess of V factor is present in the medium.

The following experiments were carried out to throw light on the origin of V factor:

Experiment 2.—The relation of the symbiosis phenomenon to hæmatin and V factor.

The basal medium used was agar made with bacto-peptone, but containing no meat and not cleared with egg. Hæmatin and yeast were added as before. The sloped tubes were inoculated diffusely with a strain of *B. influenzæ* and then along one extreme edge with *Staphylococcus aureus.*

	Growth of *B. influenzæ.*		Growth of *B. influenzæ.*
Agar + 0	0	+ staphylococcus	0
„ + hæmatin	0	„	symbiosis
„ + yeast	0	„	0
„ + hæmatin + yeast	++	„	diffuse

This experiment suggests that the symbiotic growth is due to a production of V by the staphylococcus in the presence of hæmatin. This type of growth is not apparent when V is already present in the medium.

Experiment 3.—The production of V factor in a medium from growth of *Staphylococcus aureus.*

A flask of bacto-peptone water, inoculated with staphylococcus and incubated for 48 hours, was filtered through a Berkefeld N candle and tested as follows:

Peptone-water—	Growth of *B. influenzæ.*	Staphylococcus filtrate—	Growth of *B. influenzæ.*
+ 0	0	+ 0	0
+ hæmatin	0	+ hæmatin	++
+ yeast	0	+ yeast	0
+ hæmatin + yeast	++	+ hæmatin + yeast	++

The filtrate of the culture thus contains the V factor which has been derived from the growth of the coccus.

Experiment 4.—The relation of the production of V factor to the life of the producing organism.

As is well known, V factor is often obtained by boiling yeast with water, but the proportion of yeast to water in this case is very large, and it is possible that the V extracted is obtained not so much from the yeast cells as from the extracellular products present in the commercial sample. A quantity of yeast was, therefore, washed by suspension in salt solution, collected upon a filter and thrown into acetone. The precipitated mass of yeast so obtained was dried and then extracted with water in the usual proportion. Although no exact estimation of the yield of V was made, nevertheless this factor appeared to be present in the usual quantity, and so must be supposed to have been extracted from the dead organisms.

On the other hand, experiments with staphylococcus indicate that this

organism when present in small quantity has no stimulating action on the growth of *B. influenzæ* except when alive:

A mass of *Staphylococcus aureus* was centrifuged and thrown into acetone. The acetone was decanted, the deposit rinsed in ether and heated in dry air to 100° C. for 1 hour. This dry powder was rubbed up in agar in quantities sufficient to give a slight opacity. Hæmatin and yeast were added as before.

		Growth of *B. influenzæ*.			Growth of *B. influenzæ*.
Agar	+ 0	0	+	dead staphylococci	0
,,	+ hæmatin	0	+	,,	0*
,,	+ yeast	0	+	,,	0
,,	+ hæmatin + yeast	++	+	,,	++

* Usually after 24 hours a colony of staphylococcus develops, having survived the acetone and heating, and is surrounded by a well-marked symbiotic growth of *B. influenzæ*.

The living staphylococcus is thus the only source of V factor.

The same result is obtained as follows:

			Growth in subcultures.
Peptone-water	+ hæmatin	+ 0	0
,,	,,	+ staphylococcus, living	++
,,	,,	+ do., boiled 2 minutes	0
,,	,,	+ staphylococcus from acetone	0
,,	,,	+ do., boiled 2 minutes	0
,,	,,	+ yeast	++

The growth of *B. influenzæ* was determined by subculture owing to the opacity of the tubes from the added cocci.

The conclusion may be drawn from these experiments that the production of V factor by the staphylococcus is almost entirely a function of the living organism. No doubt, if large quantities of cocci were boiled with a small quantity of water, as when a yeast extract is made, then V would also be demonstrable in the extract from the dead bodies of the cocci. In the presence of blood-pigment, the symbiosis effect is produced by the diffusion of V factor from a foreign colony in which it has been synthesised by the living organism.

This production of V factor would seem to be within the power of practically all bacteria to a greater or less extent. A large number of varieties have been tested by Kristensen (1922), with results which indicate that different species produce the same V factor, though in different amounts. On the other hand, *B. influenzæ* is an almost unique exception to this rule. As first pointed out by Davis (1921[3]), this organism has no power of stimulating the growth of influenza bacilli to produce a "symbiosis phenomenon," and so must be devoid of the power of synthesising V factor. This phenomenon was utilised by Davis as a test for influenza bacilli. With the present cultures it was found that neither *B. influenzæ* nor the Koch-Weeks bacillus had any stimulating action upon *B. influenzæ*, while the strain of *B. hæmoglobinophilus canis* had a marked effect (*cf.* Kristensen, 1922). This result is to be expected from the demonstrated growth requirements of these bacteria. Since *B. influenzæ*

demands the addition of V factor, it is not to be expected that it would synthesise it. On the other hand, *B. hæmoglobinophilus canis* does not require V factor to be added, probably because it supplies it itself.

It may be suggested that it is this failure to synthesise V factor which is the outstanding feature of *B. influenzæ* separating it from other bacteria, rather than a requirement for V factor in growth.

CRITERIA FOR INCLUSION IN THE HÆMOGLOBINOPHILUS GROUP.

From the preceding discussion it follows that the leading characters of *B. influenzæ* are—first, an absolute dependence upon an iron-containing blood-pigment for growth, and secondly, a lack of ability to synthesise V factor. Both of these characters are also exhibited by the Koch-Weeks bacillus, while the absolute dependence upon blood-pigment is shared by *B. hæmoglobinophilus canis*, without, however, the inability to synthesise V factor.

The absolute dependence of these three organisms upon blood-pigment or upon animal or vegetable cell products which have a similar (catalytic) action seems to be sufficiently unique to allow of their inclusion in one group, but until the nature of this growth requirement is more definitely known it would appear undesirable to coin a new name to replace that of "hæmoglobinophilic." This group must clearly be sub-divided into two parts, the first containing *B. influenzæ* and the probably identical Koch-Weeks bacillus, both of which are incapable of synthesising the V factor, and the second embracing *B. hæmoglobinophilus canis*, which does not suffer from this disability. The other organisms included by the Society of American Bacteriologists in the "genus Hemophilus" should not be classified with the hæmoglobinophilic bacteria.

I have had no opportunity to examine strains of "hæmolytic hæmoglobinophilic" bacteria, but according to Kristensen (1922) these organisms are not always absolutely dependent upon blood-pigment for growth. On the other hand, Rivers (1922), using methods similar to those recorded here, has found that the growth requirements of the hæmolytic bacilli are the same as those of *B. influenzæ*.

CONCLUSIONS.

B. influenzæ, Koch-Weeks bacillus and *B. hæmoglobinophilus canis*, being absolutely dependent for growth upon blood-pigment or some substance with a similar action, may be classified together as a special "hæmoglobinophilic" group. This group is capable of sub-division into two sub-groups, the first containing *B. influenzæ* and the Koch-Weeks bacillus, and the second *B. hæmoglobinophilus canis*. The former are incapable of synthesising the V factor, while the latter is capable of so doing. Other bacteria described as "hemophilic" should not be classified with the hæmoglobinophilic group.

I have to thank Mr. C. B. Goulden, Ophthalmic Surgeon to the Hospital, for selecting the cases of conjunctivitis for me.

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THE EFFECT OF LIGHT ON THE ORGANISM.

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From the Imperial Cancer Research Fund, London.

Received for publication August 7th, 1923.

THE striking results of heliotherapy, especially in surgical tuberculosis and in rickets, contrast with our ignorance of the physiological action of light on the organism. Heliotherapy is as yet purely empirical and based on clinical observations. In this connection it may not be out of place to pay tribute to the pioneer work of Dr. O. Bernhard, of St. Moritz (Switzerland). His observations, which date from 1902, on the remarkable therapeutic effect of sunlight on the healing of wounds, generally led to the application of heliotherapy to cases of surgical tuberculosis. His observations are given in detail in his book 'Sonnenlichtbehandlung in der Chirurgie,' 1917, where an interesting and detailed account is given of the history of heliotherapy and of the general biological effects of light. He also states clearly there in 1917 that light has an important effect on the formation of bone, and attributes to its absence an essential part in the ætiology of rickets. He gives as an instance the seasonal distribution of rickets in a little mountain village (Bivio) on the Julier Pass, 1776 metres above sea-level, where the winter lasts nearly nine months. He found there that the children born in autumn, who had to be kept indoors in the dark houses with their narrow windows during the nine months of winter, nearly all developed rickets in varying degree, while children born in spring, whom the parents carried with them into the fields during the summer, escaped.

In the following attempt to investigate some aspects of the physiological action of light on the organism, we were guided by the known effects of the more powerful radiations from X-rays and radium, and the curious similarity which exists between these effects and those of vitamin deficiencies. It is known that radiations from X-rays and radium in moderate doses produce a lymphopenia, while very small doses produce a lymphocytosis. It is also one of the few observed physiological effects of light that it produces a lymphocytosis (Clark, 1922). We had found (Cramer, Drew and Mottram, 1922) that large doses of radiations from radium produced a decided fall in the number of platelets, and an even more decided thrombopenia had been observed by Fabricius-Möller (1922) as the result of massive doses of X-rays. As with lymphocytes, this atrophic effect could be replaced by a stimulating effect, by applying very small doses of radiations from radium; this produced a transitory increase in the number of platelets (Mottram, 1923[1]). Since, therefore, the platelets, like the lymphocytes, are susceptible to the influence of these radiations, it seemed possible that they might also be susceptible to the action of light. The point was tested by two different lines of investigation. Rats were kept in the dark since birth, and, after the effect of the absence of light on the platelets and other tissues had been determined, they were exposed to ultra-violet light. Since we had found previously that the number of platelets could be greatly diminished by a deficiency in the vitamin A, and increased by an abundant supply of this vitamin, the animals had to be kept on a diet which was constant with reference to its content in vitamin A, and which, moreover, contained a minimal supply of vitamin compatible with the absence of disease. In another set of experiments the effect of exposure to light was studied on rats, in which a great diminution in the number of platelets was produced by keeping them on a diet free from vitamin A. If light had a stimulating effect on the formation of blood-platelets, the exposure to light should tend to counteract the effect of the vitamin A deficiency. This set of observations was carried out in 1922 before it had been found that the onset of the general effects of vitamin A deficiency, such as arrest of growth and development of bacterial infections, could be delayed by exposure to light. A preliminary account of our results was given at a discussion on the physiological action of light (Cramer, 1922[1]).

EXPERIMENTAL.

Rats which have been kept in a dark room since birth do not show any obvious signs of ill-health. They grow approximately at the same rate as rats kept in a well-lighted animal room and fed on the same diet. Nevertheless they do differ from normal rats, and this difference manifests itself morphologically by an abnormally low number of blood-platelets. In our normal laboratory stock of rats kept on a diet of bread and water, boiled maize and rice the average number of blood-platelets in a normal rat lies roughly between 800,000 and 900,000 per cmm., while the number of red blood-corpuscles lies between 9 and 10 millions per cmm. The normal platelet : red cell ratio is therefore approximately 1 : 11.

The following table (Table I) gives the number of platelets and red cells

in seven rats belonging to two litters, A and B, respectively. These two litters were born in a spacious room from which all daylight was excluded. Occasionally they received light for a few minutes from an ordinary electric bulb, which was switched on only whenever the room had to be lit for the feeding and weighing of the rats. They were kept in a deep wooden box with a lid of perforated zinc and fed on the ordinary laboratory diet mentioned above. The first counts were made when they were about three to four months old and had reached a weight of from 70 to 100 grm. The table shows that in six out of the seven rats examined the number of platelets was

Table 1

N° of Rat and Colour	Exposure to Mercury vapour lamp	Length of Exposure	Number of Red Cells per c.m.m. in millions	Number of Platelets per c.m.m.	Platelet Red Cell RATIO	Date of Count	Weight in Grms
Litter A., born 6·6·22							
481	Before	—	8·1	340,000	1 : 23	15/9	75
White	After	14 days 3′ each day	8·2	610,000	1 : 13	4/10	92
483	Before	—	7·1	370,000	1 : 19	24/10	67
White	After	25 days 5′ each day	8·8	770,000	1 : 11	22/11	75
484	Before	—	6·6	280,000	1 : 24	4/10	70
Grey	After	18 days 5′ each day	9·0	510,000	1 : 17	24/10	85
Litter B., born 4·6·22							
489	Before	—	8·9	890,000	1 : 10	4/10	100
White	——	—	—	——	——	—	—
490	Before	—	8·8	370,000	1 : 24	18/9	82
Wild + White	After	14 days 3′ each day	8·6	660,000	1 : 13	4/10	107
491	Before	—	8·2	346,000	1 : 24	4/10	87
Wild	After	18 days 3′ each day	9·1	552,000	1 : 17	24/10	95
493	Before	—	7·2	460,000	1 : 15	24/10	100
Wild	After	25 days 5′ each day	9·0	710,000	1 : 13	22/11	120

greatly subnormal. In one rat only (No. 489) the count gave normal figures. It is necessary to point out that the results were obtained with rats kept on a diet of known low vitamin content, namely, on our ordinary laboratory diet, which, though adequate to enable the rats to grow and to breed, is poor in vitamins, and produces a condition of "vitamin underfeeding" (Cramer, 1922[2]). On a vitamin-rich diet the stimulating effect of the fat-soluble vitamin counteracts the absence of light.

After the first count had been made the rats with a subnormal number of platelets were exposed to the light of a mercury vapour lamp for 3 to 5 minutes each day for periods varying in different animals from 14 to 25 days. The arrangement for exposure to light was as follows: The rat was placed in a large wooden box, one side of which had been cut away and replaced by wide-meshed wire netting. The mercury vapour lamp was placed outside the wire

netting and illuminated the whole box, in which the animal could move about freely. The counts were made in some cases on the same day as the last exposure but four hours later; in other cases two days after the last exposure, so that any temporary effects of exposure to light are excluded. The table shows in every case a very decided increase in the number of platelets, which was greatest in the two rats (Nos. 483 and 493) which had received the longest exposure to light. In these two rats the number of platelets after light treatment approximated the normal. In three rats (Nos. 483, 484, 493) a slight anæmia had developed before the light treatment. This anæmia disappeared after exposure to light.

It follows from these observations that *light has a stimulating effect on the formation of blood-platelets.* This conclusion was tested by studying the

Table 2

Vitamine A DEFICIENCY Total length of time in Weeks	Rat 530 Number in Thousands per c.m.m of		Rat 531 Number in Thousands per c.m.m. of	
	Red Cells	Platelets	Red Cells	Platelets
1	9.840	750	8.920	890
	LIGHT		NO LIGHT	
7	8.720	728	8.800	546
	NO LIGHT		LIGHT	
11	9.120	666	9.640	830

effect of light on the platelets of rats kept on a diet deficient in the fat-soluble vitamin A. We had found previously that on such a diet the number of platelets shows a progressive fall. If it be true that light had a stimulating effect on the formation of platelets, it should be possible to delay or counteract this thrombopenia of vitamin A deficiency by exposing rats kept on a diet deficient in vitamin A to ultra-violet light. Three such series of observations were carried out. In such experiments comparison can be made only if rats are used which have been bred in the laboratory and kept since birth on a known diet of a constant vitamin content. We have shown in several papers that rats kept on a vitamin-rich diet since birth resist a subsequent vitamin deficiency over a much longer period. This greater resistance manifests itself in the longer period which elapses before growth is arrested and before obvious pathological lesions develop (xerophthalmia, septic glands), and also in the slower fall in the number of platelets. For an experiment of this kind it is therefore essential to exclude as much as possible differences in resistance due to the preliminary dietary history of the animal.

In our three series a comparison was made, therefore, in each series

between rats not only of the same weight but also of the same litter which had been kept on a constant diet since birth.

Table 3

Vitamins A DEFICIENCY Total length of time in Weeks	Rat 442		Rat 443		Rat 444	
	LIGHT TREATMENT AFTER 7TH WEEK				NO LIGHT TREATMENT	
	Number in Thousands per c.m.m of		Number in Thousands per c.m.m of		Number in Thousands per c.m.m of	
	Red Cells	Platelets	Red Cells	Platelets	Red Cells	Platelets
7	9.880	340	8,760	415		
9	9.000	401	7880	250	5.480	210
12	10.880	729	9800	780	7.480	240
15	9.160	575	7.520	380		
17	9.600	657	8840	670		

LIGHT

The first two series of observations were made on rats from litters kept since birth on a vitamin rich diet. In one series (rats 530 and 531, Table II) one rat was exposed to light from a mercury vapour lamp almost from the beginning of the vitamin A deficiency for the first 7 weeks, the other being used as a control and receiving no light treatment. After this period the conditions were reversed. The results show that exposure to ultra-violet light, when applied early, delays the onset of thrombopenia; when applied later, after a thrombopenia has set in, the number of platelets increases again and may reach a normal level. It should be noted that in our previous observations on the effect of vitamin A deficiency

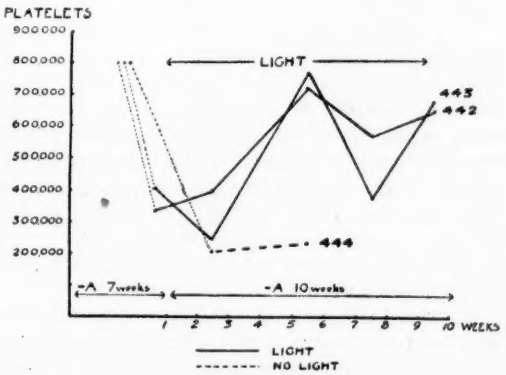

FIG. 1.

alone, the fall in the number of platelets was always progressive: after having reached a low level the number of platelets might become stationary, but never showed a decided increase.

In the second series (Nos. 442, 443, 444, Table III and Fig. 1) three rats from a litter kept on a vitamin-rich diet were placed on a vitamin A free diet. After 8 weeks, when all the rats had developed a deep thrombopenia, two of the rats (Nos. 442 and 443) received exposure to ultra-violet light, the third being kept as control and receiving no light treatment. In this third rat the deep thrombopenia was maintained, while in the other two the platelets showed a decided increase, so that even after 17 weeks of vitamin A deficiency

Table 4

Vitamins A DEFICIENCY Total length of time in Weeks	Rat 446 Number in Thousands per c.mm of Red Cells	Rat 446 Platelets	Rat 447 Number in Thousands per c.mm of Red Cells	Rat 447 Platelets	Rat 448 Number in Thousands per c.mm of Red Cells	Rat 448 Platelets
1	10.040	1.070	9.400	610	9.040	650
	LIGHT		NO LIGHT		NO LIGHT	
4	8.080	705	7.720	380	7.320	575
	LIGHT		LIGHT			
7	8.920	706	9.040	460		
	NO LIGHT					
16	7.440	297				

the number approximated 700,000 per c.cm. One of these rats developed the typical eye lesion in the nineteenth week while still under the light treatment. In this experiment, which was actually our first attempt to study the effect of ultra-violet light, the mercury vapour lamp was used at first in its original armature, which emitted only a comparatively narrow cone of light from the centre of the quartz tube. After 8 weeks of light treatment the armature was removed so that the light from the whole length of the tube illuminated the cage in which the rats were exposed. It is possible that the fluctuations in the earlier part of the experiment are due to the imperfect lighting arrangement then used.

The third series (Table IV) was carried out on rats from a litter which had been kept since birth on our ordinary laboratory diet. This diet, though containing vitamins, is so poor in these substances as to produce "vitamin underfeeding," which manifests itself in a slightly atrophic condition of the

intestinal mucosa and hastens the development of the more profound atrophic lesion when vitamin A is completely withheld. The animals of this litter (Nos. 446, 447, 448) were so susceptible to vitamin A deficiency that of three rats which were put on the vitamin A deficient diet together, the two rats which did not receive any light treatment until the fifth week of the experiment developed the typical eye lesion within 6 weeks. The only rat which escaped was No. 446, which received ultra-violet light from the beginning of the experiment till the ninth week. It actually increased in weight after the seventh week and never developed the typical eye lesion. It died after 20 weeks of pneumonia and enteritis. This is the first time that we have observed a rat from a vitamin-underfed stock to survive more than 10 weeks of vitamin A deficiency without developing the typical eye lesion or septic glands. The usual period is 6 to 7 weeks. Table II shows how the number of platelets in this rat was maintained at an almost normal level for the first 7 weeks of the experiment while the rat was receiving 5 minutes' exposures to ultra-violet light daily. The same table shows that rat 447 of the same litter had developed a profound thrombopenia as early as the fourth week. As the result of exposure to ultra-violet light, which began after the fourth week, the thrombopenia was arrested, and, in fact, the platelets showed a slight increase; xerophthalmia set in during the sixth week. The third rat (No. 448) developed the eye lesion in the fifth week—that is to say, within a week after the light treatment had begun—and died in the seventh week.

We find, then, that vitamin A and light have a similar effect in so far as the blood-platelets are concerned, although it does not necessarily follow that this effect is produced in the same way; both the intake of abundant vitamin A by the mouth and the application of light to the whole animal lead to an increase in the number of platelets, while in the absence of vitamin A from the food, or in the absence of light, the number of platelets diminished. But there is this important difference between these two agencies—vitamin A and light—that in the absence of vitamin A from the food the animal cannot live; sooner or later its health obviously declines, the intestinal mucous membrane atrophies, the animal ceases to grow, and eventually bacterial infections set in, which are usually localised either in the eyes or in the lymph-glands. None of these changes appear in animals growing up in darkness as long as they receive even a small supply of vitamin A. But such animals, when subjected to vitamin A deficiency, decline in health more rapidly than rats brought up in a well-lit room. This difference is brought out most distinctly when the rats have been kept on a diet not rich in vitamins. Thus three rats from a litter born in a dark room (13 . 11 . 22) and kept in this room on our ordinary vitamin-poor laboratory diet until they had reached a weight of 60 gm., 70 gm. and 80 gm. respectively, were compared with three rats 4 weeks younger (born 14 . 12 . 22) and weighing 50 gm. each, from a litter kept in our well-lit animal room on the same diet. Both sets of rats were placed on a vitamin A free diet and kept, the one set in the dark room, the other set in the ordinary animal room. Two rats from the "darkness" litter developed xerophthalmia after 4 weeks and the third rat after 5 weeks, the three rats from the control "light" litter only after 7 weeks, although they were

actually younger. This experiment was repeated with the same result. This difference between litters kept in light and in darkness is less distinct when the rats are kept on a diet rich in vitamins previous to the deficiency period. The stimulating effect of vitamin A on platelets and the functional development of the intestinal mucosa counteracts the atrophic effect of darkness on the platelets. Thus three rats from a "darkness" litter kept on such a vitamin-rich diet have been kept in a dark room for 19 weeks on a vitamin A free diet. Three rats of the same age from a "light" litter were kept as control on the same diet but in the well-lighted animal room. One rat of the "darkness" litter developed the eye lesion and septic glands in the neck after 7 weeks. This is the first time that we have observed a rat from a vitamin-rich stock to develop the typical infections after so short a period of vitamin A deficiency. The other two and the three control rats of the "light" litter remain, during 17 weeks of vitamin A deficiency, without any obvious sign of localised infections, and, in fact, looked quite normal. The typical eye lesion then made its appearance in all five rats within 10 days.

There is some indication in our stock of rats kept on our vitamin-poor laboratory diet that there are seasonal variations in their resistance to vitamin A deficiency. As a rule such rats develop the typical eye lesion with great regularity after 6 to 7 weeks of vitamin A deficiency. The experiments are usually carried out in sets of three rats kept in one cage, and if the eye lesion appears first in one rat it is, as a rule, within 7 to 10 days that it appears in the other two rats. It has happened repeatedly, however, that the eye lesion appeared in a whole set of three rats with exceptional rapidity 4 to 5 weeks afterwards. In going back over the protocols it was found that this occurred only in experiments carried out between December and March. This statement refers only to rats kept on our vitamin-poor laboratory diet. Our animal room, which is situated on the top of the building, is exceptionally well lighted, receiving light from large windows along two sides of the room and also from the top. It is kept warm at a fairly constant temperature day and night throughout the winter, so that cold can be excluded as the determining factor. Although not every set shows this diminished resistance to vitamin A deficiency during the winter months, the experimental results with rats kept in darkness suggest that the seasonal variations in the resistance to vitamin A deficiency are related to variations in the amount of daylight.

While absence of light diminishes the resistance of rats to vitamin A deficiency, the application of ultra-violet light increases their resistance to this deficiency. This was shown clearly by Miss Hume (1922), and by Goldblatt and Soames (1922), who studied the effect of light in vitamin A deficiency with the object of obtaining evidence as to the curative action of light in rickets. They found that rats kept on a vitamin A free diet will live longer if receiving exposures to ultra-violet light at the same time. In the end, however, they also decline in health and develop the typical symptoms of vitamin A deficiency. They conclude, therefore, that the action of light cannot be explained by assuming that there is a photosynthesis of vitamin A in the cells of the skin. Powers, Park and Simmonds have obtained similar results.

MODE OF ACTION OF LIGHT.

Two years ago we drew attention to the surprising fact that vitamin deficiency and radiations from X-rays and radium produce similar effects (Cramer, Drew and Mottram, 1921). Further investigations have established the truth of this generalisation. It may be noted as an interesting fact that very small doses of these powerful radiations have a stimulating effect on the very tissues the functional activity of which they inhibit if large doses are given. They produce in small doses a lymphocytosis and a transient thrombocytosis instead of the lymphopenia and the thrombopenia produced by large doses. It is therefore possible that light in large doses, especially ultra-violet light, may have an inhibiting instead of a stimulating effect. In this connection it is interesting and important to note that clinically it has been found that excessive exposure to sunlight produces a hæmorrhagic diathesis (Hausmann, 1923), which may in lung tuberculosis lead to hæmorrhages. Experimentally, Levy and Gassul (quoted from Clark, 1922) found in mice as the result of prolonged exposure to ultra-violet light great congestion in spleen, liver and lungs. In the lungs the congestion was so great that red corpuscles had actually passed through the capillary walls into the alveoli. We have pointed out that the similarity between the action of two such different agencies as radiations and vitamin deficiencies is not readily compatible with the generally accepted conception of the mode of action of vitamins which postulates that the life of every cell is dependent on the presence of vitamins. On the basis of that conception it is even more difficult to understand how a physical agent such as light should be able to counteract a dietetic deficiency. We have in a previous paper (Cramer, 1923) developed a different conception of the mode of action of vitamins, which allows of a ready explanation of this apparently paradoxical antagonism between light and vitamin A deficiency. Our view of the mode of action of vitamins may be briefly re-stated as follows:

MODE OF ACTION OF VITAMINS.

Vitamins are substances present in the food in variable quantities which have a drug-like stimulating effect on specific tissues, resembling in this respect the hormones of the endocrine organs. These specific tissues are in part dependent for their full functional development on the continued presence of these "food hormones," as the vitamins may be called, in the food, so that in the absence of these "food hormones" atrophic changes occur in these tissues. Thus in the absence of vitamin A the intestinal mucous membrane atrophies. A further atrophic change is the diminution in the number of blood-platelets. Quite recently an atrophic change similar to that affecting the intestinal mucosa has been described by Yudkin and Lambert (1923) as occurring in the para-ocular glands and accompanying the characteristic eye lesion. It is not necessary to discuss here how these lesions are related to each other. We will only point out that a similar change in the intestinal mucosa is produced by exposure to radium and X-rays, and the observations of Mottram (1923[2]) show that the primary effect is a disturbance in the secretion of mucus by the

epithelial cells, which allows the intestinal bacteria to come into intimate contact with the mucosa. A similar deficient secretion of mucus occurs in vitamin A deficiency, and in a recent paper by one of us we published a figure of a mucous gland of the cæcum of a rat in vitamin A deficiency filled and distended with bacteria while showing an almost complete absence of goblet cells. Under the condition of our experiments, where the rats are kept in wooden boxes with a perforated zinc lid and are therefore not exposed to any great extent to the direct effects of daylight, as they would be, for instance, in a wire cage, the atrophy of the intestinal mucosa and the thrombopenia proceed *pari passu*. These lesions are the cause of the general symptoms of vitamin A deficiency—namely, cessation of growth, invasion of bacteria and diminished resistance to certain types of bacterial infections—and the rate at which these lesions develop determines the onset of the general symptoms. Of these two lesions, one, the thrombopenia, can be counteracted by exposure to light, which stimulates the production of platelets. It follows, therefore, that any condition which tends to diminish the number of platelets—such as absence of light—when applied before the animal is subjected to vitamin A deficiency, must handicap the organism in its reaction against the deficiency and hasten the decline. Conversely conditions which stimulate the production of platelets—such as exposure to light—must assist the organism in resisting the general effects of vitamin A deficiency and delay the decline. By thus referring the effects of vitamin deficiency to specific pathological lesions, it is possible to give an explanation of the apparently paradoxical antagonism between two such different agencies as light and a dietetic factor.

We have in a previous paper made the suggestion that the blood-platelets represent a defensive mechanism against bacterial infection, and given evidence from our own observations as well as from those of other workers in support of this view. The fact that a factor such as light, which stimulates the production of platelets, tends to delay the onset of bacterial infections in vitamin A deficiency, is further evidence in support of this view. It is also interesting to note that Yudkin and Lambert quote Wason and Smith as having found a diminution in the bactericidal titre of the serum of deficiently fed animals.

ON THE ORIGIN OF PLATELETS.

The problem of the manner in which light produces its effect in increasing the number of platelets is bound up with the problem of the origin of platelets, of which latter problem we know unfortunately very little. If the platelets are formed exclusively in some internal organ—and it is held by some observers that they are formed by the megakaryocytes of the bone-marrow—then the action of light on platelet formation, which can only affect the cells of the skin or the underlying capillaries and lymphatics, can only be explained by an indirect photosynthetic effect. We would then have to assume that these peripheral cells under the influence of light form a chemical substance which passes into the blood and stimulates the production of platelets in sites such as the bone-marrow which are remote from the direct action of light. In order to obtain information on the origin of platelets we have examined the bone-marrow both in smears and in paraffin sections from normal rats, rats

suffering from a vitamin A deficiency and rats recovering from vitamin A deficiency. The material was fixed in sublimate acetic acid, stained with Giemsa, and differentiated with mixtures of acetone and xylol in varying proportions. We have never been able to obtain evidence which would indicate the formation of platelets from the megakaryocytes. In fact the bone-marrow was always remarkably poor in platelets. On the other hand the spleen is frequently very rich in platelets, which lie scattered through the parenchyma of the spleen. In one instance a section through an artery and a vein lying side by side showed the vein crowded with platelets while the artery showed very few. Veins from lymph-glands in the subcutaneous tissue have also often been found to contain numerous platelets. In some preparations from rats recovering from vitamin A deficiency under the influence of cod-liver oil, the veins show a sprouting of the cytoplasm of the endothelial cells with platelets lying embedded in the cytoplasm. Platelets are also very numerous in the lymphatics of the mesentery of the rat. These appearances, which are put forward at present as representing merely the results of a preliminary investigation, suggest that platelets are formed by the endothelial cells of lymphatics and blood-vessels. This view of the origin of platelets agrees with the findings of Bedson (1922) that antiplatelet serum, which is highly specific for platelets, has also a toxic effect on the endothelium of the blood-vessels. If platelets have a general origin of this kind, then the effect of light might be attributed to a local stimulating action of light on the protoplasm of the endothelial cells in the peripheral capillaries and lymphatics. Light is, however, not essential for the formation of platelets. For platelets are present in considerable numbers in the blood of the embryo.

Variations in the number of platelets are bound to have an effect on the chemical composition of the blood. For platelets which represent an extremely sensitive and delicate type of protoplasm must be conceived as constantly undergoing disintegration and pouring their contents into the plasma. This may account for the chemical changes which have been found to occur in the blood as the result of exposure to light.

SUMMARY.

Rats born and kept in darkness frequently show a diminution in the number of blood-platelets as compared with rats kept on the same vitamin-poor diet in a well-lighted room. This thrombopenia is sometimes associated with a definite anæmia. Exposure of such animals to a mercury vapour lamp increases the number of blood-platelets to normal, and, if an anæmia was present, increases the number of red cells. Rats kept in darkness do not exhibit intestinal lesions such as are found in vitamin A deficiency, nor do they develop the bactericidal infections (xerophthalmia, septic lymph-glands) characteristic of that deficiency. They do, however, develop these conditions sooner when placed on a vitamin A deficiency diet than rats kept in a well-lighted room.

Rats kept on a vitamin A free diet develop a progressive thrombopenia and an atrophy of the intestinal mucosa. Exposure to a mercury vapour lamp if applied from the beginning of the deprivation of this vitamin, delays the

thrombopenia. If applied after a thrombopenia has set in, it will increase the number of platelets. Such exposures to light, however, do not prevent the eventual onset of the atrophy of the intestinal mucosa which results from the vitamin A deficiency.

The conclusion is drawn that light is a stimulus to the formation of blood-platelets. This explains the partial antagonism between vitamin A deficiency and exposure to light: light can counteract the one lesion—thrombopenia—produced by vitamin A deficiency, but cannot prevent the eventual atrophy of the intestinal mucous membrane. Exposure to light can therefore delay but cannot prevent the eventual cessation of growth and the eventual onset of bacterial infections resulting from the vitamin A deficiency. Absence of light accelerates the onset of these conditions. Light is, however, not an essential condition for the formation of platelets, and its absence can be counteracted so far as the platelets are concerned by an abundant supply of vitamin A.

The fact that conditions which stimulate the production of platelets will delay the development of certain types of bacterial infections is further evidence in support of the view that platelets represent a mechanism against bacterial infection. The mode of action of light and the origin of platelets are discussed.

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DIPHTHERIA TOXOID AS AN IMMUNISING AGENT.

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Received for publication August 8th, 1923.

In a previous paper (Glenny, Allen and Hopkins, 1923) methods have been described for testing diphtheria toxin-antitoxin mixtures for use in active immunisation against diphtheria. By means of the methods there described it is now possible to determine the relative antigenic values of mixtures of toxin and antitoxin of varying strength and constitution, and of modifications of toxin. An ideal immunising agent is one that presents the highest antigenic strength but does not cause any injurious effects. The object of this present paper is to show what work has been done towards producing an ideal immunising agent against diphtheria.

Most of our knowledge of the relative immunising values of different toxins has been obtained from the routine immunisation of horses for the large scale production of diphtheria antitoxin. Experience has shown that the immunising value of a batch of toxin depends upon its specific antigenic value in relation to its toxicity. In this connection, by toxicity is meant not only specific toxicity, *i. e.* the number of minimal lethal doses of diphtheria toxin present, but also non-specific toxicity due mainly to broth constituents. When we are dealing with toxin-antitoxin mixtures for human use it is necessary also to consider the amounts of horse-serum present when given to serum sensitive subjects, and also of the "pseudo" constituent in the toxin when the mixture is used to immunise a "combined" (pseudo and positive) Schick-reactor. The specific antigenic value of a toxin or of a toxin-antitoxin mixture depends upon the number of free binding units, whether of toxin or toxoid. For the purposes of this paper the term "toxoid" will be used in its general sense for any modification of toxin that will still combine with antitoxin but is no longer fatal to guinea-pigs. That toxoid is of equal specific antigenic value to toxin has long been known; in 1904 one of us produced diphtheria antitoxin containing over 1000 units per c.c. from a horse injected only with diphtheria toxin so modified that the M.L.D. for a guinea-pig of 250 gm. weight was 0·3 c.c. and the L 0 dose 0·5 c.c.; a few years later similar high value antitoxin was obtained from a horse injected with modified toxin, of which 5·0 c.c. would not kill a guinea-pig. The immunising value of modified toxin rich in toxoid is greater than that of toxin because, while the specific antigenic value remains the same, the specific toxicity is reduced or absent. When we are dealing, however, with hyper-immunisation, specific toxicity is of less importance, owing to the presence in the animal of sufficient circulating antitoxin to neutralise most of the specific toxin. Yet it is

interesting to note in passing that a small local injection is not all neutralised by circulating antitoxin, although the total antitoxin present in the animal may be enough to neutralise many thousand times the amount of toxin injected. The Schick reaction in man is a typical example of this; a man with five litres of blood containing $\frac{1}{100}$ unit of antitoxin per c.c. gives a Schick-positive reaction when injected with that amount of toxin which would be completely neutralised by $\frac{1}{1000}$ unit of antitoxin, *i.e.* $\frac{1}{5000000}$ of the total circulating antitoxin in the patient. Again, immunised horses containing, say, 30 litres of blood have developed diphtheria paralysis after an injection of toxin that could be completely neutralised by 1 c.c. of their blood.

The use of modified toxin in toxin-antitoxin mixtures for human use enables a far greater number of binding units, *i.e.* a greater specific antigenic strength, to be presented without any increase in specific toxicity. It may be possible shortly to use toxin so modified that it will be completely non-toxic without the addition of antitoxin. This would constitute a marked advance in the prevention of diphtheria; the presence of horse-serum in a toxin-antitoxin mixture might possibly cause reactions in serum-sensitive subjects, and possibly sensitise others not already sensitive, although no worker has as yet found evidence of sensitisation by such small doses of serum in a human subject. The use of modified toxin, however, reduces the amount of serum necessary in a mixture, because such modified toxin need not be fully neutralised; only sufficient antitoxin need be added to reduce the residual toxicity below a certain level.

The cause of the variation in non-specific toxicity of different batches of toxin is not known, nor is there any convenient test beyond that of large scale immunisation of horses. In our experiences two batches of toxin may have identical values for M.L.D., L 0 and L +, and so presumably have the same specific antigenic values, and yet differ so markedly in their true immunising property that one may be regarded as almost worthless and the other as extremely good. It is not yet known whether non-specific toxicity is due entirely to absolutely non-specific broth constituents or whether bacterial protein may play some part. In toxin-antitoxin mixtures intended for human use dilutions of the mixtures must lessen the non-specific toxicity.

The original American standard for specific toxicity of a toxin-antitoxin mixture was that 1 c.c. should cause no ill-effects in a guinea-pig, while 5 c.c. should kill in 10 days or more. Later the specific antigenic value was increased by using mixtures with more free toxin, and it was found that mixtures were safe for human use if 1 c.c. did not kill guinea-pigs in less than 10 days. A still greater improvement was made by using dilutions of more toxic mixtures. We, however, have endeavoured to increase the number of binding units in mixtures without making them any more toxic, and have continued to accept the early American standard of toxicity.

The modification of toxin used in the experiments recorded in this paper was prepared by adding 0·1 per cent. formalin to diphtheria toxin and exposing it for 4 weeks at a temperature of 37° C. before removing to the cold room. The original values of the toxin showed an M.L.D. of 0·0025 c.c.; L 0, 0·15 c.c.; and L+, 0·2 c.c. After treatment the M.L.D. was 0·2 c.c.; the L 0, 0·3 c.c.; and the L+, 1·0 c.c.

The routine method now adopted by us for testing antigenic values has already been described by us (Glenny, Allen and Hopkins, 1923). This method consists in injecting guinea-pigs with a single dose of the toxin-antitoxin mixtures or toxin under test, resting the animals for 3 weeks, and then injecting at weekly intervals with Schick doses of toxin until the animals fail to show a reaction. The number of Schick doses so given, until the animal is sufficiently immune to show no reaction, is an index of the antigenic value of the material under test, and is now termed by us the "immunity index." Thus, if a guinea-pig was injected with 1·0 c.c. of a certain toxin-antitoxin mixture, and 3 weeks later the first Schick injection gave a positive reaction, and the following week the second Schick injection showed no reaction, then the immunity index for 1·0 c.c. of that mixture would be recorded as 2.

Occasionally in experimental work we have endeavoured to determine whether a substance has any value as an antigen to diphtheria. If the antigenic value is very low many Schick injections will be needed before the immunity of the test-animal is raised to the Schick-negative level; if the substance under test has no antigenic value, the immunity index will be the same number of Schick doses of diphtheria toxin that would need to be given at weekly intervals to a normal guinea-pig in order to raise its immunity to the Schick-negative level. It is necessary, therefore, to know the effect of weekly injections of Schick doses of toxin upon normal guinea-pigs.

In all experimental work on animals we have adopted as the Schick dose of toxin the amount that is just neutralised by $\frac{1}{1000}$ unit of antitoxin. This level was adopted because it represented the binding-unit content of $\frac{1}{50}$ M.L.D. of a well-matured toxin used in our first experiments, and, as we have already pointed out (Glenny and Allen, 1922), the level of immunity detected by a Schick test depends upon the number of binding units injected and not upon the toxin content alone. It was found that the number of weekly Schick doses necessary to immunise a guinea-pig varied according to the toxin used. Table I shows that a relatively fresh toxin, of which $\frac{1}{30}$ M.L.D. was equivalent to $\frac{1}{1000}$ unit of antitoxin, gave an average index of 31, while the shortest time in which a guinea-pig injected weekly with this dose became immune was 20 weeks. An old matured toxin, containing $\frac{1}{50}$ M.L.D. to the Schick dose, gave an average index of 12, while the lowest record for the toxin was 9. With the modified toxin one guinea-pig, when given only $\frac{1}{200}$ M.L.D. each week, was sufficiently immune to give no reaction to the sixth weekly injection, while the majority were immune by the time they received the seventh injection.

TABLE I.

Toxin number.	Description.	Volume injected.	Fraction of M.L.D.	Average index.	Lowest index.
J. 3284	Fresh toxin	0·0001 c.c.	$\frac{1}{30}$	31	20
J. 1915	Old toxin	0·0004 c.c.	$\frac{1}{50}$	12	9
Y.M.B. 101	Modified toxin	0·001 c.c.	$\frac{1}{200}$	7	6

If so few injections of so small an amount of "toxoid" will immunise a guinea-pig, we may hope in the course of time so to improve the methods of

diphtheria prevention that a single dose of modified toxin will act both as a Schick test and as an immunising agent.

The antigenic values of one or two injections of modified toxin acting as a primary stimulus were measured by the method already described, and the results are recorded in Table II.

TABLE II.

Dose.	No. of experiments showing an immunity index of—						
	1	2	3	4	5	Over 5.	Total.
Two injections of 0·01 c.c. .	—	4	2	—	—	1	7
One injection of 0·05 ,, .	—	15	9	5	5	5	39
,, ,, 0·1 ,, .	2	—	—	—	—	—	2
,, ,, 0·2 ,, .	1	1	—	—	—	—	2

The general condition of the test animals has a great influence upon the apparent immunity index of an antigen. A good antigen may occasionally show a poor index; thus in Table II two injections of 0·01 c.c. modified toxin have yielded a bad index once in seven experiments. A large number of tests have been made at different times upon the immunity index for a single dose of 0·05 c.c. because we have found that this is a useful critical dose, sufficiently effective as a stimulus to yield a very good index of 2 on many occasions, while at the same time the dose is not excessive. The minimal dose that will constantly yield a good index under good conditions will frequently yield a poor index if the test animals are subject to adverse conditions; the immunity index of a larger dose is not so easily affected. For this reason we use a dose of 0·05 c.c. of this modified toxin when testing the conditions affecting the production of immunity. The experiments quoted in the table refer only to those guinea-pigs that were not injected with other substances to affect the rate of production of immunity, but include all control animals and those of varying weight. Eight out of the ten experiments showing an immunity index of 4 or 5 occurred in a single group of animals injected at a time when feeding conditions were not good, and, consequently rate of growth was slow and infection rate high. The antigenic value of a single dose containing 0·05 c.c. of modified toxin is at least as good as that of the majority of batches of toxin-antitoxin mixtures which we have tested. Such a dose of unneutralised modified toxin is not more specifically toxic than a toxin-antitoxic mixture, contains from one-tenth to one-twentieth of the non-specific constituents, and in addition contains no horse-serum. Four guinea-pigs were injected with higher doses, and it will be seen from Table II that three out of four guinea-pigs injected with from ½ to 1 M.L.D. were Schick negative when first tested 3 weeks later, while the fourth animal was negative at the second test made 4 weeks after the primary stimulus.

The action of toxoid as a primary stimulus has already been described by one of us (Glenny and Sudmersen, 1921), when it was pointed out that "three weeks after a single dose of from 0·5 c.c. to 2·0 c.c. of an old formalinised toxin (L 0 dose about 1·0 c.c.; M.L.D., 5·0 c.c.), a number of guinea-pigs survived 2 M.L.D. of toxin without any local reaction." One guinea-pig that

had received four injections of toxoid over a long period of time reached an antitoxic titre of 8·5 units per c.c.

In the preparation of toxin-antitoxin mixtures from unmodified toxin there is very little margin between mixtures that are too toxic and mixtures so over-neutralised that they are of very low antigenic value. With modified toxin the differential region between L 0 and L + is so great that there is a very large margin of safety in the preparation of mixtures.

TABLE III.

Dose injected: 0·1 c.c. of a mixture containing—	Number of experiments showing an immunity index of—						
	1	2	3	4	5	Over 5.	Total.
0·1 c.c. antitoxin (27 units) + 10·0 c.c. modified toxin (approx. L 0)	—	2	4	—	—	—	6
0·1 „ „ 14·0 „ „	—	2	—	—	—	—	2
0·1 „ „ 20·0 „ „	—	3	1	—	—	—	4
0·1 „ „ 25·0 „ „	—	1	2	—	—	—	3
0·1 „ „ 30·0 „ „ (approx. L+)	1	6	—	—	—	—	7
0·1 „ „ 42·0 „ „	—	2	—	1	—	—	3
0·1 „ „ 50·0 „ „	—	—	2	—	—	—	2

Table III shows that mixtures may be prepared containing from 10 to 50 c.c. of modified toxin to the same amount (0·1 c.c.) of a certain antitoxin, and with all mixtures throughout this range the immunity index for 0·1 c.c. of the mixture is as good as that for 1·0 c.c. of the majority of the mixtures prepared from unmodified toxin. Several of the mixtures were tested at lower doses; thus the mixture containing 42 c.c. of modified toxin and 0·1 c.c. of antitoxin was injected in doses of 0·01 c.c. into 3 guinea-pigs; 2 gave an immunity index of 2 and 1 of 4. In this mixture the toxoid was only neutralised to the extent of about 20 per cent. The specific toxicity of the mixture was such that when diluted 1 in 10 the mixture would correspond with the U.S.A. standard for toxin-antitoxin mixtures, yet a high index is given when diluted 1 in 100.

The effect of modified toxin as a secondary stimulus to actively immune rabbits is shown by Table IV.

TABLE IV.

Rabbit.	Antitoxic value before injection in units per c.c.	Volume of modified toxin injected.	Maximum value after injection in units per c.c.	Time interval.
10	0·08	5·0 c.c.	12·0	8 days.
42	0·0005	0·05 c.c.	2·75	6 „
55	0·0005	0·05 c.c.	3·75	6 „
99	0·015	0·1 c.c.	1·75	11 „
120	0·0005	0·05 c.c.	0·04	8 „
121	0·0005	0·05 c.c.	0·03	6 „
148	0·0005	0·05 c.c.	9·0	10 „
151	0·005	0·05 c.c.	4·5	8 „

Further work is being carried out along the following lines:

(1) Purification of toxin by methods of concentration which increase the number of binding units per milligramme of nitrogen about 40 fold.

(2) Using toxin so further modified that it is not toxic even in large doses.

(3) Investigation of the spectrum of partial neutralisation in relation to antigenic values.

Our thanks are due to our medical colleagues Drs. O'Brien, Eagleton and Okell for the information that a mixture of modified toxin and antitoxin (that containing 20·0 c.c. of "toxoid" to 0·1 c.c. of antitoxin and diluted 1 in 10) has given exceptionally promising results when employed for human immunisation.

SUMMARY.

The value of a diphtheria toxin for immunising purposes increases as toxin changes into toxoid (or toxone).

Toxins can be changed into toxoid by the action of formalin.

Such modified toxin containing 1·5 M.L.D. per L 0 and 5 M.L.D. per L+ has been used for the production of toxin-antitoxin mixtures showing a high immunity index.

It is hoped by further reduction in toxicity and by concentration to produce an immunising agent of far greater efficiency than any yet employed for protection against diphtheria.

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